

**Development of Acetic-acid Tolerant *Zymomonas mobilis* Strains
through Adaptation**

A Thesis

Presented to

The Academic Faculty

By

Yun Wang

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Chemical and Biomolecular Engineering

Georgia Institute of Technology

August 2008

**Development of Acetic-acid Tolerant *Zymomonas mobilis* Strains
through Adaptation**

Approved by

Dr. Rachel Chen
School of Chemical & Biomolecular Engineering
Georgia Institute of Technology

Dr. Sankar Nair
School of Chemical & Biomolecular Engineering
Georgia Institute of Technology

Dr. Athanassios Sambanis
School of Chemical & Biomolecular Engineering
Georgia Institute of Technology

Date Approved: April 24, 2008

ACKNOWLEDGMENTS

First and foremost, I would like to thank my advisor Dr. Rachel Chen for her help. She gave me lots of great ideas for my experiments and my study. She also taught me lots of new knowledge. Under her supervision, I successfully completed my research and thesis.

Second, I would also like to thank my committee members for their guidance and time. They gave me great ideas for my thesis.

Third, I would also like to thank Dr. Shin and Dr. Mao. They help me a lot with my experiments. Especially, at the beginning of my research, they taught me how to use different equipments in the lab. They made my first day in the lab much easier. They also gave me great suggestions to improve the experiments.

Finally, I would also like to thank all other students in my lab. They are nice and kind. They gave me lots of help during my lab times. And I am happy to be part of this wonderful team.

TABLE OF CONTENTS

ACKNOWLEDGMENTS	iii
LIST OF TABLES	vii
LIST OF FIGURES	viii
SUMMARY	xi
CHAPTER 1 INTRODUCTION	1
1.1 Ethanol production by <i>Zymomonas mobilis</i>	1
1.2 Previous research to reduce the effect of acetic acid on <i>Z. mobilis</i> in fermentation	7
1.2.1 Tolerant strain by genetic modification	7
1.2.2 Optimizing the fermentation conditions	8
1.3 Objectives	10
1.4 Approaches	10
CHAPTER 2 <i>Zymomonas mobilis</i>	13
2.1 Advantages of <i>Z. mobilis</i>	13
2.2 Physiology of <i>Z. mobilis</i>	14
2.3 Production of ethanol by <i>Z. mobilis</i>	19
CHAPTER 3 ACETATE TOXICITY AND TOLERANCE OF ACETIC ACID	23
3.1 Toxicity observed in microorganisms	23
3.2 Mechanism of acetic acid toxicity	28
3.3 Tolerant mechanism	34
CHAPTER 4 EXPERIMENTS	42
4.1 Microorganism and culture maintenance	42
4.2 Medium	42

4.2.1 Seed medium.....	43
4.2.2 Fermentation medium	43
4.2.3 Medium for other inhibitors experiments	43
4.2.4 Solid medium for agar plates	44
4.3 NTG mutagenesis	44
4.4 Colony screening	44
4.5 Mutant characterization	45
4.6 Analytical methods	46
4.6.1 Optical density (O.D.).....	46
4.6.2 Ethanol measurement.....	47
4.6.3 Glucose and acetic acid measurement	49
CHAPTER 5 ADAPTIVE MUTATION	50
5.1 Adaptive mutation and mutant selection	50
5.1.1 Adaptive mutation by gradually increasing the acetic acid concentration	53
5.1.2 Adaptive mutation by reducing pH.....	62
5.2 NTG mutagenesis	67
CHAPTER 6 MUTANT CHARACTERIZATION.....	76
6.1 The characteristics of wild type strain and mutants obtained by adaptive mutation	76
6.2 Comparison of mutants developed by adaptive and NTG mutagenesis	90
6.3 Ethanol production characteristics of mutants.....	97
6.4 Other inhibitors	101
CHAPTER 7 CONCLUSIONS AND RECOMMENDATIONS.....	105

7.1 Conclusions.....	105
7.2 Recommendation	106
7.3 Future research.....	107
REFERENCES	109

LIST OF TABLES

Table 1.1 Important traits for ethanol production	3
Table 1.2 A comparison of attributes for ethanol production by <i>Zymomonas</i> and yeast....	4
Table 1.3 Composition of common agricultural lignocellulosic feedstock	6
Table 3.1 Relative concentrations of Asps in AAB during growth in the presence of 10 g/L acetic acid	36
Table 3.2 <i>E.coli</i> genes whose relative expression level increases after treatment with sodium acetate.....	38
Table 4.1 O.D. values and its natural logarithm value at different times	47
Table 5.1 Summary of the mutants developed by adaptive mutation.....	67
Table 6.1 Lag phases (h) of five strains at different pHs and acetic acid concentrations.	88
Table 6.2 Specific growth rates (h^{-1}) of five strains at different pHs and acetic acid concentrations	89
Table 6.3 Lag phases (h) of six strains at different pHs and acetic acid concentrations ..	95
Table 6.4 Specific growth rates (h^{-1}) of six strains at different pHs and acetic acid concentrations	96
Table 6.5 Ethanol production characteristics of two mutants in different fermentation conditions (% of theoretical number)	101
Table 6.6 O.D. value and specific growth rate of fermentation in the presence of different inhibitors	103
Table 6.7 Ethanol production of two strains in the presence of different inhibitions (% of theoretical number)	104

LIST OF FIGURES

Fig. 1.1 Ethanol production process from Biomass	2
Fig. 2.1 Entner-Doudoroff pathway in <i>Zymomonas mobilis</i>	15
Fig. 2.2 Effect of ethanol on total fatty acid composition of <i>Z. mobilis</i> ATCC 10988. (A) cells allowed to accumulate ethanol during fermentation; (B) cells harvested in exponential phase in the presence of added ethanol	17
Fig. 2.3 <i>Zymomonas mobilis</i> engineered for metabolism of xylose	18
Fig. 2.4 Procedure of simultaneous saccharification and fermentation (SSF).....	21
Fig. 3.1 Effect of acetic acid on the growth of <i>Z. mobilis</i> ATCC 29191 at pH 5	24
Fig. 3.2 Effect of acetic acid on the maximum specific growth rate of <i>Z. mobilis</i>	25
Fig. 3.3 Effect of acetic acid on the glucose metabolism by <i>Z. mobilis</i> at pH 5.....	25
Fig. 3.4 Effect of acetic acid on ethanol yield	26
Fig. 3.5 Effect of pH and acetic acid on growth	26
Fig. 3.6 Permeability of the cell plasma membrane to HAc – the undissociated form of acetic acid; and the concentration of HAc as a function of the pH of the medium (insert)	30
Fig. 3.7 Comparison of ³¹ P NMR spectra of <i>Z. mobilis</i> ZM4 (pZB5) at 2.5 min after addition of 277 mM glucose at 30°C and pH 5.5 in the presence (A) and absence (B) of 10.9 g/L of sodium acetate. 1, sugar phosphates; 3, extracellular phosphate; 4, triethyl phosphate as the internal standard; 5, NDP; 6, NAD and NADP; 7, UPD sugars; 8, β-NTP	34
Fig. 3.8 A cell model with five fluxes in utilization and detoxification of acetic acid by <i>R. eutropha</i>	40
Fig. 4.1 Agar plate for colony screening.....	45
Fig. 4.2 Natural logarithm of O.D. value as a function of time	47
Fig. 4.3 Ethanol calibration line.....	48
Fig. 4.4 Acetate calibration line.....	49

Fig. 4.5 glucose calibration line.....	49
Fig. 5.1 Adaptive mutation process	51
Fig. 5.2 The sequence of mutation.....	52
Fig. 5.3 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of twelve colonies from adaptive mutation with 0.2% acetic acid (Horizontal lines represent the average of the twelve colonies analyzed.)	55
Fig. 5.4 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of twelve colonies from adaptive mutation with 0.5% acetic acid (Horizontal lines represent the average of the twelve colonies analyzed.)	57
Fig. 5.5 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of thirteen colonies from adaptive mutation with 1.0% acetic acid (Horizontal lines represent the average of thirteen colonies analyzed.).....	58
Fig. 5.6 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of six colonies from adaptive mutation with 1.2% acetic acid (Horizontal lines represent the average of six colonies analyzed.)	60
Fig. 5.7 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of six colonies from adaptive mutation with 1.4% acetic acid (Horizontal lines represent the average of six colonies analyzed.)	62
Fig. 5.8 Specific growth rate and ethanol concentration for nine colonies from adaptive mutation at pH 5.5 in the presence of 1.0% acetic acid (Horizontal lines represent the average of nine colonies analyzed.).....	64
Fig. 5.9 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of eight single colonies from adaptive mutation at pH 5.0 in the presence of 1.0% acetic acid (Horizontal lines represent the average of eight colonies analyzed.)	66
Fig. 5.10 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of four single colonies from NTG mutagenesis at pH 6.0 and in the presence of 1.4% acetic acid (Horizontal lines represent the average of four colonies analyzed.).....	69
Fig. 5.11 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of four single colonies from NTG mutagenesis at pH 6.0 and in the presence of 1.6% acetic acid (Horizontal lines represent the average of four colonies analyzed.).....	71
Fig. 5.12 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of four single colonies from NTG mutagenesis at pH 5.5 and in the presence of 1.4% acetic acid (Horizontal lines represent the average of three colonies analyzed.)	73

Fig. 5.13 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of four single colonies from NTG mutagenesis at pH 5.5 and in the presence of 1.6% acetic acid (Horizontal lines represent the average of four colonies analyzed.).....	75
Fig. 6.1 Growth curves at pH 6.0 in the absence of acetic acid.....	77
Fig. 6.2 Growth curves at pH 6.0 in the presence of 1.0% acetic acid	78
Fig. 6.3 Growth curves at pH 6.0 in the presence of 1.2% acetic acid	79
Fig. 6.4 Growth curves at pH 6.0 in the presence of 1.4% acetic acid	80
Fig. 6.5 Growth curves at pH 5.5 in the absence of acetic acid.....	81
Fig. 6.6 Growth curves at pH 5.5 in the presence of 1.0% acetic acid	82
Fig. 6.7 Growth curves at pH 5.5 in the presence of 1.2% acetic acid	83
Fig. 6.8 Growth curves at pH 5.5 in the presence of 1.4% acetic acid	84
Fig. 6.9 Growth curves at pH 5.0 in the absence of acetic acid.....	85
Fig. 6.10 Growth curves at pH 5.0 in the presence of 1.0% acetic acid	85
Fig. 6.11 Growth curves at pH 5.0 in the presence of 1.2% acetic acid	86
Fig. 6.12 Growth curves at pH 5.0 in the presence of 1.4% acetic acid	87
Fig. 6.13 Growth curves at pH 6.0 in the presence of (A) 1.4% and (B) 1.6% acetic acid	91
Fig. 6.14 Growth curves at pH 5.5 in the presence of (A) 1.4% and (B) 1.6% acetic acid	93
Fig. 6.15 Growth curves at pH 5.0 in the presence of (A) 1.4% and (B) 1.6% acetic acid	94
Fig. 6.16 Ethanol production of ZM5510 at different pHs and concentrations. (A) pH 5.0, 1.4% acetic acid, (B) pH 5.5, 1.4% acetic acid, (C) pH 6.0, 1.4% acetic acid, (D) pH 5.0, 1.6% acetic acid, (E) pH 5.5, 1.6% acetic acid, (F) pH 6.0, 1.6% acetic acid.....	98
Fig. 6.17 Ethanol production of ZM6014 at different pHs and concentrations. (A) pH 5.0, 1.4% acetic acid, (B) pH 5.5, 1.4% acetic acid, (C) pH 6.0, 1.4% acetic acid, (D) pH 5.0, 1.6% acetic acid, (E) pH 5.5, 1.6% acetic acid, (F) pH 6.0, 1.6% acetic acid.....	99

SUMMARY

Zymomonas mobilis is one of the best ethanol producers. Its high ethanol productivity, in particular, makes it attractive as a fermenting microorganism for large scale bioethanol production from biomass feedstock. One critical barrier for this microorganism, however, is its sensitivity to acetic acid, an inhibitor present in pretreated biomass at a relatively high concentration. Acetic acid inhibits cell growth and lowers the ethanol productivity. While removal of acetic acid is technically feasible, the economics of ethanol production calls for alternative approach.

The goal of this work is to develop acetic acid tolerant *Zymomonas mobilis* for use in bioethanol production. An adaptive mutation procedure was developed. Using this procedure, several highly tolerant strains were obtained. The characteristics of these mutants were carefully studied. Compared to the wild type strain, these mutants exhibited higher specific growth rate, higher final O.D. and had significantly shorter lag phase in the presence of acetic acid, indicating superior tolerance to acetic acid.

One adapted mutant (ZM5510) was further developed by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutation. Four mutants, ZMNTG5514, ZMNTG5516, ZMNTG6014 and ZMNTG6016 were obtained. These four mutants performed no better than the best mutant from adaptive mutation, suggesting that adaptive mutation alone is sufficient to develop acetic acid tolerance in *Z. mobilis*.

This study also reveals that mutants developed for acetic acid tolerance have significantly enhanced tolerance to other biomass-derived inhibitors including formic acid and vanillin, suggesting common tolerant mechanisms.

The acetic acid tolerant strain developed in this work will be useful in ethanol production from biomass feedstock. The adaptive mutation procedure developed in this work may be applicable to other microorganisms and for other inhibitors.

CHAPTER 1 INTRODUCTION

In this chapter, the advantages of *Zymomonas mobilis* for ethanol production will be stressed, along with the bottlenecks that limit the application of *Zymomonas mobilis* in industry. In addition, a brief review of research performed in this area will be offered. The goals of this research project will then be formulated.

1.1 Ethanol production by *Zymomonas mobilis*

Fossil fuel is not renewable. As the demand of energy increases worldwide, fossil fuel is rapidly depleted. Therefore alternative sources of energy have to be evaluated to meet the global energy demand. Methane, hydrogen and ethanol are considered as potential substitutes for fossil fuels [1]. Among these three candidates, ethanol is considered to be a good choice for an alternative liquid fuel in the near term. First, it can be produced from a variety of agriculture-based renewable materials, such as sugarcane juice, molasses, potatoes, corn and barley [2]. Second, ethanol is environment friendly because it decomposes to water and CO₂ after reaction. The reaction equation is as follows [3]:

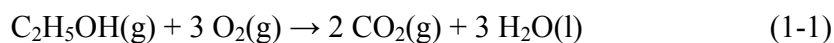


Figure 1.1 displays the process of ethanol production using biomass as the feedstock [4]. In this process, both glucose and pentose are fermented to ethanol by a microorganism.

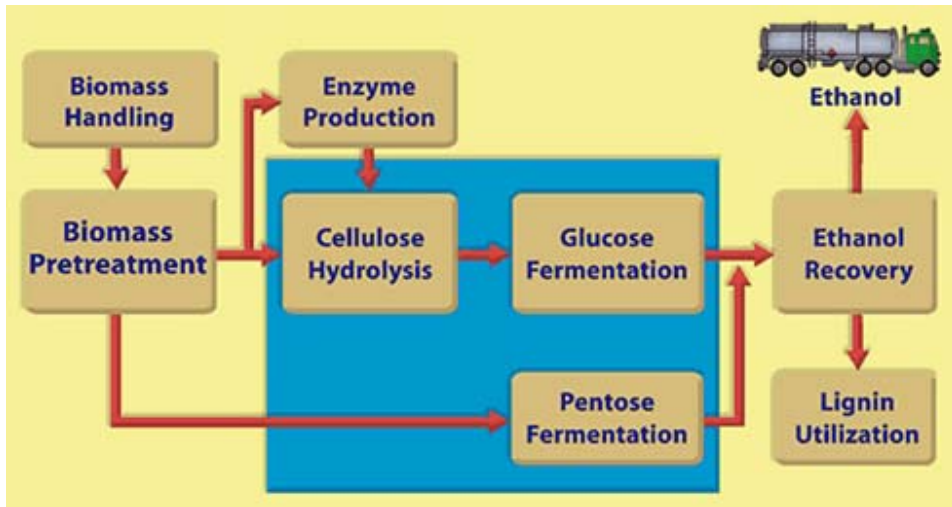


Fig. 1.1 Ethanol production process from Biomass [4]

Currently, yeast (*Saccharomyces cerevisiae*) is commonly used in the process [5]. Despite its popularity, yeast has a number of disadvantages. First, it has a limit on ethanol tolerance [6-8]. It was found that the wild type *S. cerevisiae* ATCC4123 could not grow in the presence of 93 g/l ethanol [9]. Thus, considerable researches were devoted to improve the ethanol tolerance and the ethanol tolerant mechanism of this microorganism [6-9]. Second, there is a major by-product, glycerol, during the ethanol fermentation by *S. cerevisiae* under both aerobic and anaerobic conditions. Glycerol production reduces ethanol production [10]. More seriously, under aerobic condition [11], oxygen concentration has to be controlled to minimize glycerol production [12]. This means, the concentration of oxygen has to be controlled at a certain value, which obviously increases the production cost. For example, when fermenting the 25 degrees Brix honey solution by *S. cerevisiae*, the dissolved oxygen concentration has to be controlled around 20%, because the ethanol production rate and ethanol yield decreases when the dissolved oxygen concentration is higher than 20% [13]. Third, *S. cerevisiae* has narrow substrate

utilization range. The wild type strain *Saccharomyces* is capable of fermenting galactose, glucose and mannose, but it cannot ferment the xylose found in lignocellulosic feedstock because it lacks both a xylose-assimilation pathway and adequate levels of key pentose phosphate pathway enzyme [14]. Only after genetic engineering, the recombinant strain could metabolize either xylose or arabinose [15-17].

Because of these disadvantages, a number of other microorganisms have been tested for the production of ethanol.

Table 1.1 Important traits for ethanol production [18]

Trait	Requirement
Ethanol yield	>90% of theoretical number
Ethanol tolerance	>40 g/l
Ethanol productivity	>1 g/(l*h)
Robust grower and simple growth requirements	Inexpensive medium formulation
Able to grow in undiluted hydrolysates	Resistance to inhibitors
Culture growth conditions retard contaminants	Acidic pH or higher temperatures

When choosing the microorganisms, several important traits are usually considered, including yield, ethanol tolerance, productivity, and growth requirements (Table 1.1) [18]. Among these traits, the ethanol yield is received the most attention because feedstock typically accounts for greater than one-third of the production costs [18]. If ethanol yield is high, less feedstock would be needed to produce the same amount of ethanol. Consequently, the production cost could be reduced, so high ethanol yield is imperative. Based on this requirement, *Z. mobilis*, which was found to have the highest ethanol yield

on sugar complex containing glucose [19-21], became one of the most promising microorganisms having the potential to replace yeast for ethanol production. This microorganism has been demonstrated to have ethanol yields up to 97% of the theoretical value. When compared with traditional yeast fermentation, it could achieve 5 to 10% higher yield [14, 22, 23].

Another advantage of *Z. mobilis* is its high ethanol productivity. The volumetric ethanol productivity of *Z. mobilis* could be five-fold higher than *S. cerevisiae* [22, 23]. Table 1.2 shows a comparison of various attributes between these two microorganisms based on either a batch fermentation system or a continuous cultivation with cell recycle [24]. Although the same ethanol yield was obtained by the two microorganisms, the ethanol productivity and the volumetric ethanol productivity were much higher in the process employing *Z. mobilis*.

Table 1.2 A comparison of attributes for ethanol production by *Zymomonas* and yeast [24]

Parameters	<i>Z. mobilis</i>	Yeast
Conversion of sugar to ethanol (%)	96	96
Maximum ethanol concentration (%)	12	12
Ethanol productivity rate (g ethanol/(g glucose *h)) (Batch fermentation, glucose = 10%)	5.67	0.67
Volumetric ethanol productivity (g ethanol/(l*h)) (Continuous culture with cell recycle, glucose = 10%)	200	29

Additional advantages of *Z. mobilis* for ethanol production include the high sugar tolerance, the low production cost and the ability to ferment sugar at low pH. *Z. mobilis* could grow at high concentrations of glucose (10-25%) [25]. This microorganism is also acid tolerant and could grow over a pH range of 3.5 to 7.5. So the fermentations are generally resistant to bacterial contamination. Furthermore, this microorganism could grow under both aerobic and anaerobic conditions. But aerobic growth does not result in higher cell yields or growth rates compared to anaerobic growth, so there is no need to control the addition of oxygen to maintain the cell viability, which reduces the production cost [14, 24].

Although *Z. mobilis* is better than yeast in many aspects, it has not been used commercially, despite considerable researches carried out on a lab or pilot scale. The following factors are considered most important in preventing its commercial use.

Firstly, wild type *Z. mobilis* only uses glucose, fructose and sucrose as their substrates. Since xylose is a major component of hemicellulose in most biomass feedstock (Table 1.3) [26], it is essential for a fermenting microorganism to use this sugar in ethanol production for a good product yield from biomass. Fortunately, metabolic engineering has been successfully applied to develop a *Zymomonas* strain to ferment xylose [27, 28], as well as arabinose [29]. By genetic engineering technology, engineered *Z. mobilis* could potentially use all sugars present in most biomass feedstock (as shown in Table 1.3).

Table 1.3 Composition of common agricultural lignocellulosic feedstock [26]

	Corn stover	Wheat straw	Bagasse	Cotton gin	Sugar beet pulp	Switch grass
Carbohydrate (%)						
Glucose	34.6	32.6	39.0	37.1	24.1	31.0
Mannose	0.4	0.3	0.4	1.1	4.6	0.2
Galactose	1.0	0.8	0.5	2.4	0.9	0.9
Xylose	19.3	19.2	22.1	9.4	18.2	0.4
Arabinose	2.5	2.4	2.1	2.3	1.5	2.8
Uronic acid	3.2	2.2	2.2	N/A	20.7	1.2
Non-carbohydrate (%)						
Lignin	17.7	16.9	23.1	28.8	1.5	17.6
Extractives	7.7	13.0	3.8	7.7	N/A	17.0
Ash	10.4	10.2	3.7	10.5	8.2	5.8

Secondly, *Z. mobilis* is sensitive to acetic acid, especially at low pH [30]. The toxicity of acetic acid was shown intensified during xylose fermentation [31]. The pretreated biomass by dilute-acid usually contains up to 1.5% acetic acid (w/v) due to the hydrolysis of the acetylated pentoses in hemicellulose. Before using *Z. mobilis* in industry, this inhibition problem has to be addressed.

1.2 Previous research to reduce the effect of acetic acid on *Z. mobilis* in fermentation

Previous research was carried out to address the acetic acid toxicity. In this section, these studies will be briefly reviewed. Strategies to overcome this toxicity are either genetic modification or process optimization.

1.2.1 Tolerant strain by genetic modification

1) N-methyl N'-nitro N-nitrosoguanidine (NTG) treatment

Rogers and his co-workers used this method in 1998 [32]. First, a culture of wild type *Z. mobilis* (ZM4) strain was treated with NTG. Mutants were then selected through chemostat with an increase of sodium acetate in the medium fed every 72 hours. Finally, the culture was plated to isolate the acetic acid tolerant mutant. After analysis, it was found the mutant *Z. mobilis* (ZM4/Ac^R) could produce ethanol efficiently in the presence of 20 g/l sodium acetate (equivalent to 14.7 g/l acetic acid) at pH 5.0, and the maximum specific growth rate was 0.17 h⁻¹ under this condition. This compared to the wild type *Z. mobilis* (ZM4) strain, which was inhibited significantly with 12 g/l sodium acetate (equivalent to 8.8 g/l acetic acid) and stopped to grow in the presence of 16 g/l sodium acetate (equivalent to 11.7 g/l acetic acid). Rogers and his co-workers further studied the acetic acid tolerant strain for xylose fermentation using the transformant ZM4/Ac^R (pZB5) [31]. Plasmid pZB5 contains genes associated with xylose utilization [33]. Their study found that the ZM4/Ac^R (pZB5) had the higher acetic acid tolerance than ZM4 (pZB5) during xylose fermentation. The experimental results showed that ZM4/Ac^R (pZB5) exhibited generally higher values of maximum specific growth rates and xylose uptake

rates following glucose depletion than ZM4 (pZB5) under the same conditions. For example, in batch fermentation, the xylose uptake rate of ZM4/Ac^R (pZB5) was 0.4 g/(g*h) in the presence of 12 g/l sodium acetate, 25 g/l glucose and 25 g/lxylose, whereas, no growth for ZM4 (pZB5) under this condition.

2) Recombinant DNA technology

Baumler and his co-workers proposed a method to enhance the acid tolerance in *Z. mobilis* (CP4) [34]. They cloned a portion of the *cbpA* gene from *Escherichia coli* K-12 encoding a 24 amino acid proton-buffering peptide (Pbp) via a shuttle vector. The experimental results showed that the recombinant CP4 (pJB99) had the higher acid tolerance than CP4. For example, 70.6% *Z. mobilis* (CP4) with pJB99 survived after one hour incubation in Tryptone soya broth (TSB) medium at pH 3.5, however only 17% *Z. mobilis* (CP4) strain survived under the same condition. They hypothesized that the Pbp contributed to cytoplasmic buffering, because the percentage of amino acids in Pbp capable of accepting one or more protons was greater than typical proteins. They speculated that proton-buffering peptide (Pbp) may play a role in pH homeostasis although the mechanisms of pH homeostasis in *Z. mobilis* have not been fully elucidated. Another finding from this work was that the acid tolerance was even higher when recombinant strain grew in the presence of ampicillin. However, the mechanism of “ampicillin effect” has not been understood.

1.2.2 Optimizing the fermentation conditions

1) Removal of acetic acid from pretreated biomass

Acetic acid could be removed from hemicellulosic hydrolysates by ion-exchange resins and ion exchange membranes [35]. The experiments were carried out to remove the acetic acid from DI water and hemicellulosic hydrolysate by these two methods. The results showed that the acetic acid capacity of ion exchange membrane was higher than ion exchange resins. And it was also better for concentrating the eluted acetic acid. So ion exchange membrane may provide an efficient means of removing acetic acid from biomass hydrolysates in the future. But the cost of this process has to be determined later [36].

2) Finding optimum fermentation conditions for the recombinant *Z. mobilis*

The purpose here is to reduce the inhibition of acetic acid on an industrial scale by changing the fermentation conditions, especially for the xylose fermentation, which is more sensitive to acetic acid than glucose fermentation. The National Renewable Energy Laboratory (NREL) had considered a bioconversion process, named “simultaneous saccharification and fermentation (SSF)” for converting lignocellulosic biomass to ethanol on an industrial scale [37]. The recombinant strain ZM39676:pZB4 was investigated using this process at different acetic acid concentrations and pHs. Their experimental data showed that the cell mass decreased with decreasing pH or increasing acetic acid concentration, and the maximum cell mass was obtained at pH 6.0 at any acetic acid concentration. But the xylose utilization was affected by the value of pH value*acetic acid concentration. For example, in the presence of 1% (w/v) acetic acid, the maximum xylose utilization rate was at pH 5.5. This means the optimum condition had to be found considering both biomass and sugar utilization. So changing the conditions could also reduce the effect of acetic acid on fermentation process.

1.3 Objectives

As stated above, *Z. mobilis* has several advantages for ethanol production, as well as two disadvantages. The goal of this research is to develop acetic acid tolerant strain for ethanol fermentation.

An adaptive mutation will be developed and acetic acid tolerant strain will be selected and fully characterized in terms of growth behavior and ethanol fermentation characteristics.

Additionally, acetic acid tolerant mutants will be tested for possible cross-protection against other representative inhibitors commonly present in pretreated biomass.

1.4 Approaches

In this research, we proposed a new method - adaptive mutation to develop acetic acid tolerant strains

John Cairns first proposed the concept in 1988. He said "when populations of single cells are subject to certain forms of strong selection pressure, variants emerge bearing changes in DNA sequence that brings about an appropriate change in phenotype" [38, 39]. The most significant evidence of adaptive mutation is *E. coli* strain FC40 [40]. This strain cannot utilize lactose but if put this strain in the medium with the lactose as the only carbon and energy source, it could revert to lactose utilization. This means the strain, which could grow in the medium with lactose, must have some mutations inside the cell. The experiments also showed that the rate of the mutation related to the lactose utilization was greater than mutations in other parts of the genomes of these *E. coli* cells, which means the useful changes for surviving were preferred.

In 1991, the term “adaptive mutation,” was coined. The term was used to describe a process that “produces mutations specific to the selective pressure but does not produce mutations that are useless or deleterious” [41].

In contrast to random mutations, such as UV or chemical mutagens, adaptive mutation tends to produce only useful mutations [42]. In other words, the cells appear to have a mechanism to prevent the useless genetic changes. The evidence for this conclusion presented in Cairns’s experiment was that only mutants with the selected phenotype appeared. Other scientists, such as Benson, Hall, independently showed that mutants did not arise if they were not useful [42].

Adaptive mutation has been used to increase the rate of alcohol production in *Z. mobilis* (CP4) [43]. In this experiment, culture was serially transferred in the medium in the presence of 10^{-5} M allyl alcohol for ten times, followed by serially transferring in the medium in the presence of 5×10^{-5} M or 7×10^{-5} M allyl alcohol for eighteen months. Finally, a single colony was selected. Allyl alcohol was chosen as selective pressure here because it could be oxidized to a toxic product, acrolein, by alcohol dehydrogenase, acting in the opposite direction as ethanol production during fermentation. If the strain could grow in the presence of allyl alcohol, this strain could show higher ethanol production without allyl alcohol. The batch fermentation results showed that the mutant *Z. mobilis* (CP4) had the higher cell density and higher ethanol productivity than its parent strain under the same condition. The ethanol concentration for the mutant *Z. mobilis* (CP4), at which cells stopped to grow, was also higher than that of the parent strain.

The disadvantage of adaptive mutation is that, it is poorly understood and it has unpredictable nature of its outcome. Additionally, it is time consuming; finally, it is only applicable to those phenotypes that the selective pressure can be easily applied to.

Adaptive mutation is suitable for this work because selective pressure could be applied by gradually increasing acetic acid concentration and those cells, that are tolerant could be easily selected as they outgrow those less tolerant.

However one premise to use this approach is that the cell growth and ethanol production is coupled. It could not be a successful method if the ability of acetic acid tolerance compromised ethanol production.

If this method is successful for acetic acid, it could be used for other inhibitors in the pretreated biomass, such as vanillin, hydroxybenzoic acid, hydroxymethylfuraldehyde [44].

CHAPTER 2 *Zymomonas mobilis*

In this chapter, the advantages of *Z. mobilis* for ethanol production are outlined. These advantages are related to the physiology of the strain. For this reason the physiology of *Z. mobilis* is reviewed emphasizing those most relevant to ethanol production. Finally, current state of fermentation technology, both batch and continuous processes are reviewed.

2.1 Advantages of *Z. mobilis*

Z. mobilis is a Gram-negative bacterium. It was first isolated in tropical countries from alcoholic beverages such as the African palm wine [45]. The optimum pH of *Z. mobilis* is 5.5-7.0 and the optimum temperature is 25-31 °C [46]. *Z. mobilis* is a unique bacterium and offers a number of advantages for ethanol production over the existing ethanol producing microorganisms. Researches of this microorganism for ethanol production date back to 1970s.

Several attributes of *Z. mobilis* are particularly attractive for large-scale ethanol production. These include [47-49]:

- 1) Tolerance to high concentrations of sugar (up to 400g/l).
- 2) Tolerance to high concentration of ethanol (up to 130g/l).
- 3) Low yields of biomass and high yields of ethanol (up to 1.9 mole ethanol per mole glucose utilized under anaerobic conditions) on certain sugars.
- 4) High specific rates of sugar uptake.
- 5) No requirement of controlled addition of oxygen during fermentation.

- 6) Amenability to genetic manipulations.

2.2 Physiology of *Z. mobilis*

Z. mobilis is one of the few facultative bacteria degrading glucose by the Entner-Doudoroff (ED) pathway anaerobically [24, 50, 51], which is commonly used by strictly aerobic microorganisms such as *Pseudomonas*. This pathway firstly degrades sugars to pyruvate, and the pyruvate is then fermented to produce ethanol and carbon dioxide as the only products (Figure 2.1) [24]. The rate-limiting steps in the sugar metabolism are the conversion of glucose-6-phosphate to 6-phosphogluconate and of 3-phosphoglycerate to 2-phosphoglycerate [24]. Relatively large amounts of pyruvate decarboxylase (pdc) and alcohol dehydrogenase (adh) appear to be critical for rapid ethanol formation [50]. The ED pathway is a homo-ethanol fermentation pathway. It yields only half as much ATP per mole of glucose as the Embden-Meyerhoff-Parnas (EMP) pathway [52]. As a consequence, *Zymomonas* produces less biomass than yeast and more carbon is funneled to fermentation products, which leads to high ethanol yield. Also, as a consequence of the low ATP yield, *Zymomonas* maintains a high glucose flux through the ED pathway. All the enzymes involved in fermentation are expressed constitutively, and fermentation enzymes comprise as much as 50% of the cells total protein. So *Z. mobilis* has a high rate of sugar uptake.

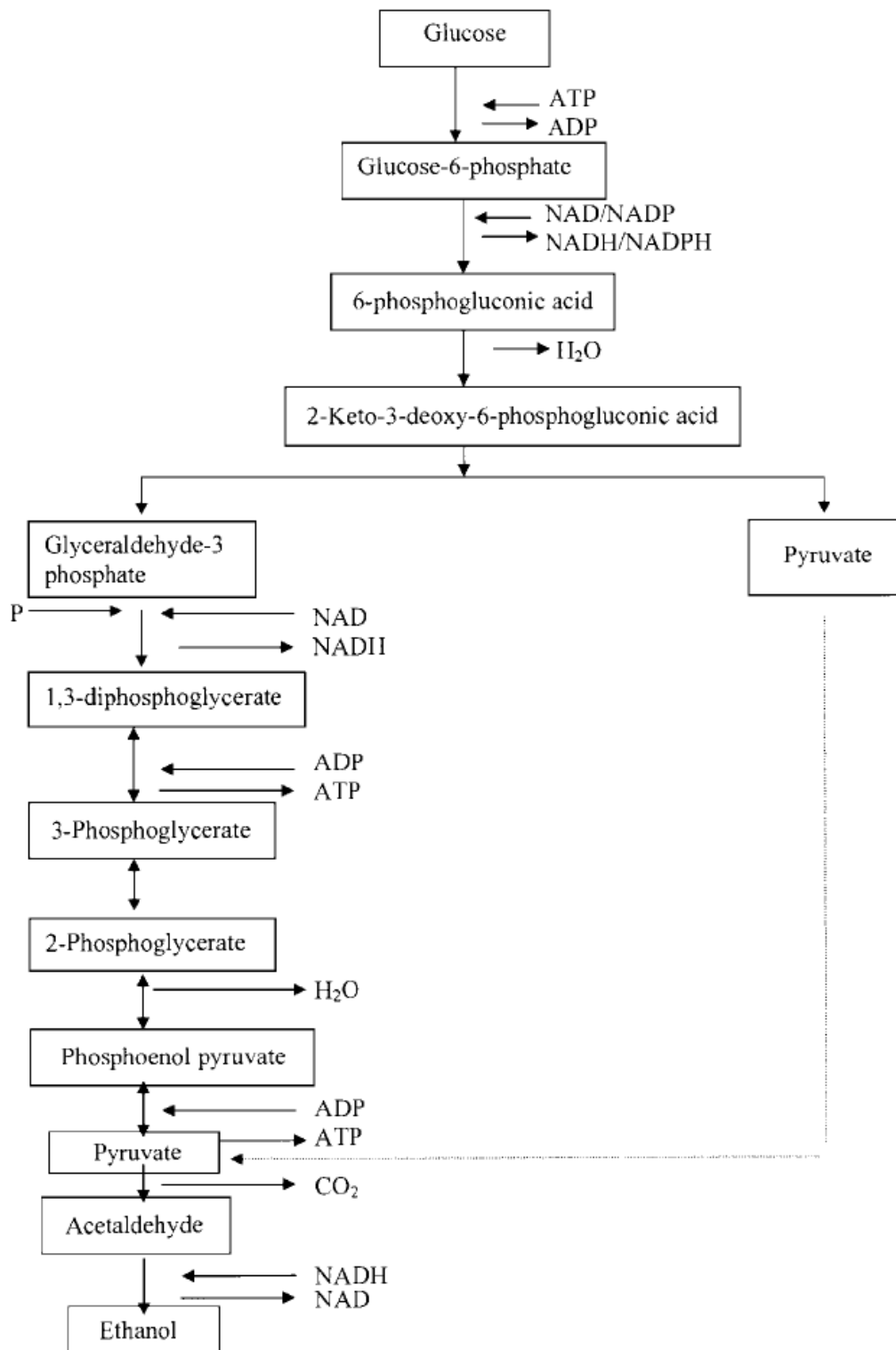


Fig. 2.1 Entner-Doudoroff pathway in *Zymomonas mobilis* [24]

In *Z. mobilis*, D-glucose is transported by facilitated diffusion instead of active transport system. It is believed that this facilitated diffusion sugar transport system of *Zymomonas*, coupled with the high expression of pyruvate decarboxylase and alcohol dehydrogenase genes, is responsible for its high glucose tolerance [14, 23, 53]. Since conversion of glucose to ethanol by this microorganism proceeds rapidly and the glucose is transported by diffusion, the extracellular osmotic pressure of the glucose solution may rapidly be balanced by corresponding intracellular sugar concentrations. So concentrated glucose solutions are not inhibitory to the ED pathway enzymes.

The high ethanol tolerance of *Z. mobilis* is related to the characteristics of its membrane. The cell membrane of *Z. mobilis* has acquired altered fatty acid profile to counteract the adverse effects of ethanol. In *Z. mobilis*, over 60% of the total fatty acids are vaccenic acid (Figure 2.2) [54]. Vaccenic acid is a long chain phospholipid, which could increase the surface area for hydrophobic and van der Waals interaction and decrease the polarity within the hydrophobic core of the membrane. Therefore, vaccenic acid is important to restore the primary permeability barrier of the cell during growth in the presence of ethanol. Another important characteristic of the cell membrane of *Z. mobilis* is that its fatty acid composition remains relatively unaffected by ethanol (Figure 2.2). This is because that *Z. mobilis* is very limited in metabolic capability, only producing CO₂ and ethanol as fermentation end products from glucose. To survive in the presence of ethanol, this microorganism may have to synthesize only the fatty acid composition most advantageous for growth and survival in the presence of ethanol. This microorganism has evolved well to survive in the environment in the presence of high concentration of ethanol, which is produced by this strain. Another reason of *Z. mobilis*'s

high ethanol tolerance is that its plasma membrane contains hopanoids [47]. The concentration of hopanoid in this strain is about 30 mg/g dry cell weight, which is the highest amount observed in bacteria so far. The hopanoids play an important structural role in the cytoplasmic membrane. The interactions among the hopanoid side chains give reinforcement to the membrane. Hopanoids can also counteract both condensing and fluidizing effects on the membrane. Therefore, a high and constant amount of hopanoids in membranes can establish optimal membrane stability and fluidity over a wide range of ethanol concentrations.

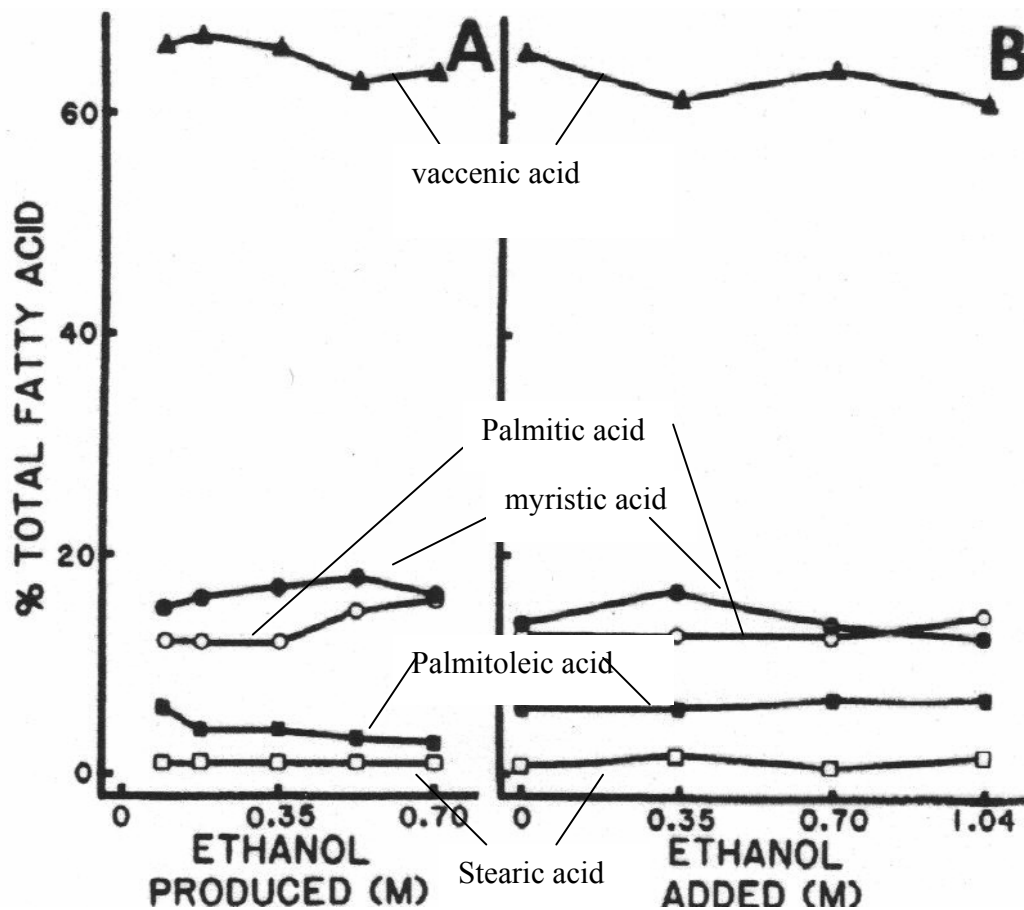


Fig. 2.2 Effect of ethanol on total fatty acid composition of *Z. mobilis* ATCC 10988. (A) cells allowed to accumulate ethanol during fermentation; (B) cells harvested in exponential phase in the presence of added ethanol [54]

Z. mobilis is a prokaryote, and it is more amenable to genetic manipulations. This is important as genetic engineering is most likely needed for strain improvement [45]. For example, by inserting the genes from other organisms, *Z. mobilis* is able to ferment xylose and other sugars [18]. In 1995, researchers at the National Renewable Resources Laboratory (Department of Energy, United States) successfully engineered strains capable of fermenting xylose by introduction and expression of four *E. coli* genes: xylose isomerase (xylA), xylulose kinase (xylB), transketolase (tktA), and transaldolase (talB) in *Z. mobilis* strains [28]. The new metabolism process of xylose is summarized in Figure 2.3. The newly introduced enzymes produced by the new genes could convert the xylose to fructose-6-P and glyceraldehydes-3-P, which was then channeled to the Entner-Doudoroff (ED) pathway for ethanol production. The ethanol yield of this strain was 86%.

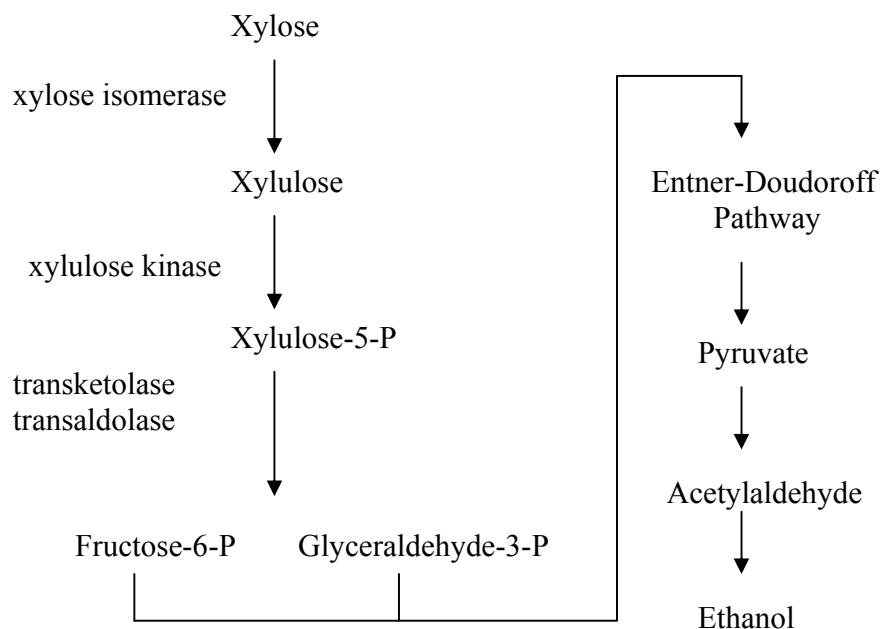


Fig. 2.3 *Zymomonas mobilis* engineered for metabolism of xylose [28]

The strategy used to engineer *Z. mobilis* for xylose metabolism was also used to construct a strain that fermented arabinose. In this case, five genes isolated from *E. coli*: L-arabinose isomerase (araA), L-ribulose kinase (araB), L-ribulose-5-phosphate-4-epimerase (araD), transketolase (tktA) and transaldolase (talB) were cloned into and expressed in *Z. mobilis* strain. This new strain could successfully ferment arabinose (25 g/l) to ethanol with a high yield (98% of theoretical value). The combination of xylose and arabinose utilizing enzyme allowed a strain to ferment both arabinose and xylose [18, 23, 27, 28].

Z. mobilis is classified as a facultative anaerobic microorganism. Cultivation is under anaerobic conditions. Yet, it tolerates oxygen quite well, which means no need to maintain strict anaerobic conditions.

Additionally *Z. mobilis* is generally regarded as safe (GRAS). So the distilled dried grain from *Zymomonas* fermentation is generally recognized as safe for use as animal fodder [23].

2.3 Production of ethanol by *Z. mobilis*

As mentioned in Chapter 1, wild type *Z. mobilis* only ferments sucrose, glucose and fructose for production of ethanol from biomass. Usually engineered strains are used. The production of ethanol from biomass by *Z. mobilis* has been studied both on a lab or pilot scale. Various reaction systems and biomass stocks have been tested.

On a lab scale, with *Z. mobilis* grown on Jerusalem artichoke juice, Toran-Diaz [55] obtained an ethanol productivity of 4.8 g/g/h, which was higher than that reported for the yeast *Kluyveromyces marxianus* by Duvnjak [56]. And they also observed that the juice

of Jerusalem artichoke could be fermented without the addition of any nutrients. Lawford *et al.* [57] demonstrated that corn steep liquor, a by-product of maize wet-milling, was a cost-effective substrate for production of ethanol by *Z. mobilis* (CP4). Torres and Baratti [58] reported that in batch fermentation, sugar (wheat starch) concentrations as high as 223 g/l could be fermented to 105 g/l ethanol in seventy hours. The yield obtained was 92%. Nellaiah *et al.* [59] revealed the strain NRRL B-4286 of *Z. mobilis* could ferment glucose, fructose, and sucrose up to a concentration of 200 g/l. NRRL B-4286 was also proved to be the best strain for fermentation of cassava starch hydrolysate [53].

On a pilot scale, a continuous system is usually used. Allais *et al.* [60] found that the volumetric productivity was 67.2 g/l/h with a final ethanol concentration of 42 g/l from 100 g/l initial sugars by continuous production of ethanol from Jerusalem artichoke Juice using ZM4F of *Z. mobilis*. Doelle [61] described a process for the continuous production of ethanol from hydrolysates of starch by *Z. mobilis* based on a single-stage fermentation. They reported a conversion yield of 92%. Sahm and Bringer-Meyer [62] described a process for the continuous production of ethanol on an industrial scale from hydrolysed wheat starch using *Z. mobilis*. They reported *Z. mobilis* produced 60 g ethanol/l over a test period of thirty-nine days.

Another unique system is solid-state fermentation. This system is economical when the feedstock is agricultural byproducts, such as sweet sorghum, corn, apple, grape, sugar cane, sugar beets, fodder beets, and Jerusalem artichoke tubers. Because these stocks have complex composition and they are insoluble, solid-state fermentation of these sources would be the best choice [53]. Because solid-state fermentation is a relatively new system, very few reports are available regarding the production of ethanol by solid

state fermentation. Amin [63] has described ethanol fermentation in solid state by *Z. mobilis* grown on sugar-beet. Ethanol yield was 0.48 g/g sugar, volumetric productivity was 12 g/l/h and final ethanol concentration was 130 g/l. The performance of *Z. mobilis* in a solid-state fermentation was good. Furthermore, Amin reported that during solid-state fermentation fewer by-products were produced compared to conventional submerged fermentation.

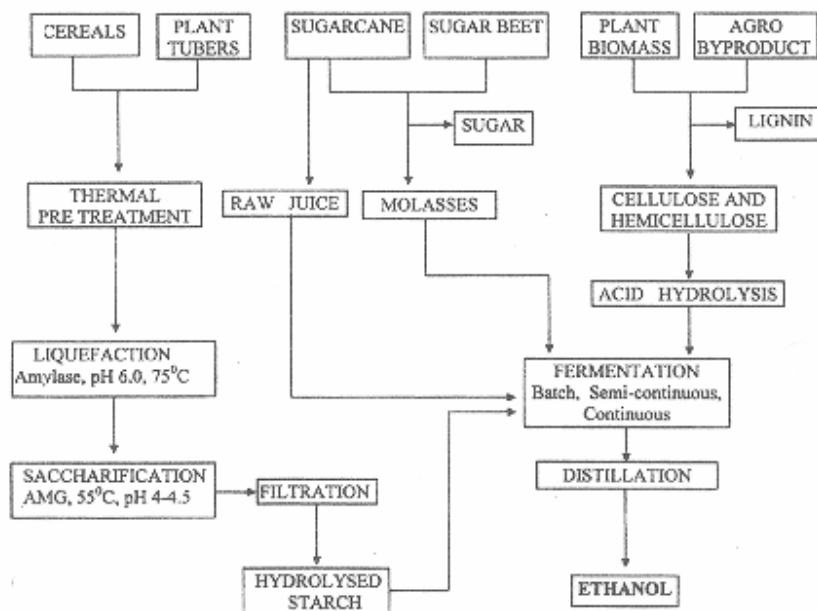


Fig. 2.4 Procedure of simultaneous saccharification and fermentation (SSF) [53]

To produce the ethanol from biomass, sometimes another microorganism capable of producing carbohydrate hydrolase is used to saccharify the polymeric substrate, besides *Z. mobilis*. The saccharified products are simultaneously utilized by *Z. mobilis* for ethanol production. This process is called simultaneous saccharification and fermentation (SSF) [53, 64]. Figure 2.4 summarizes the steps of this process [53]. Rhee *et al.* [65]

investigated various SSF processes. Ethanol production from sweet sorghum was achieved to 29.7 g ethanol/100 g dry sorghum stalks by using *Fusarium oxysporum* mixed culture with *Z. mobilis*.

CHAPTER 3 ACETATE TOXICITY AND TOLERANCE OF ACETIC ACID

In this chapter, the effect of acetic acid on *Z. mobilis*, as well as *E. coli* is reviewed, along with the effect of other biomass components. This will be followed by a brief review on current understanding of the toxicity mechanism and tolerance mechanism.

3.1 Toxicity observed in microorganisms

Ethanol is produced from pretreated biomass, which usually contains acetic acid. The acetic acid concentration from a variety of woody biomass and waste materials is in the range of 2-15 g/L. Acetic acid has high boiling point (116°C) [66], so it could not be removed during evaporation of the hydrolysis liquors [44].

Acetic acid is an inhibitor for the growth of *Z. mobilis*. Lawford carried out some experiments to investigate the effect of acetic acid at pH 5.0 (Figure 3.1 to Figure 3.2) [30]. The ethanol and glucose were measured by HPLC. The growth of the strain was measured by spectrophotometer. And the specific growth rate was calculated from the growth data. Figure 3.1 shows that 1.22 g/L acetic acid results in a two hour increase in lag time. The maximum specific growth rate (μ_{\max}) is decreased from 0.42 h⁻¹ to 0.34 h⁻¹. Figure 3.2 shows that even a small amount of acetic acid could alter specific growth rate. The growth rate is reduced to 50% in the presence of 8.3 g/L acetic acid. Figure 3.2 also shows that the response of acetic acid dosage is not linear for growth rate, and the general shape of these plots is similar to the titration of a weak acid. The curve is relatively flat in the range of 2-8 g/L, so improving the tolerance of acetic acid higher than 8.0 g/L is important. Their work found that at this pH, there was no growth observed at

concentrations of acetic acid up to 11 g/L. Acetic acid also affects the ethanol production and glucose consumption (Figure 3.3). Because the lag phase increases and specific growth rate decreases with the increase of acetic acid, the time when strain starts to consume glucose and produce ethanol in the presence of acetic acid is delayed as compared to that in the absence of acetic acid. The shape of volume productivity curves corresponds to the specific growth rate curves. However, the effect of acetic acid on the glucose-to-ethanol conversion yield is small, except when the strain does not grow at all. The ethanol yield decreases from 0.49 to 0.46 g/g in the presence of 6 g/L acetic acid. And the shape of the yield curves (Figure 3.4) matches to that of specific growth rate.

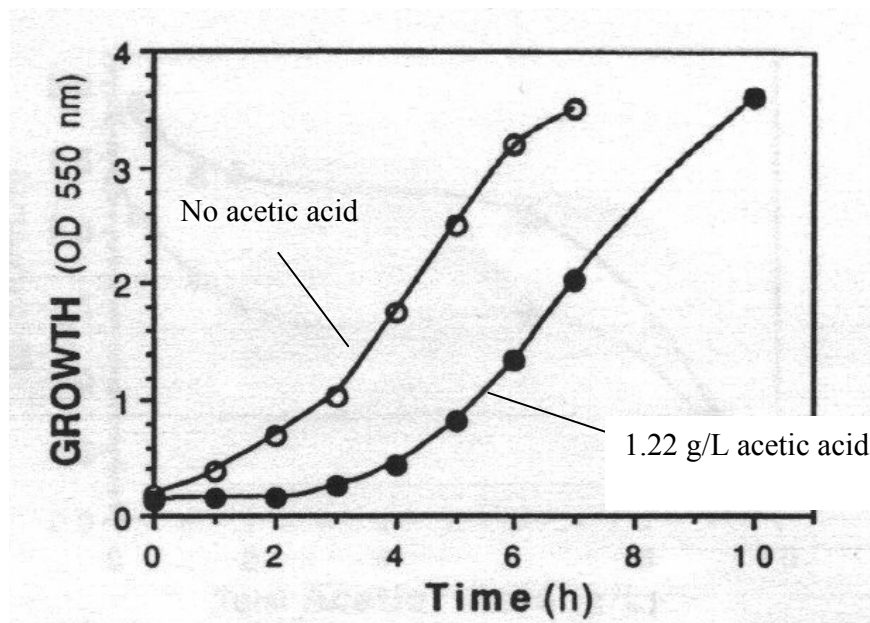


Fig. 3.1 Effect of acetic acid on the growth of *Z. mobilis* ATCC 29191 at pH 5 [30]

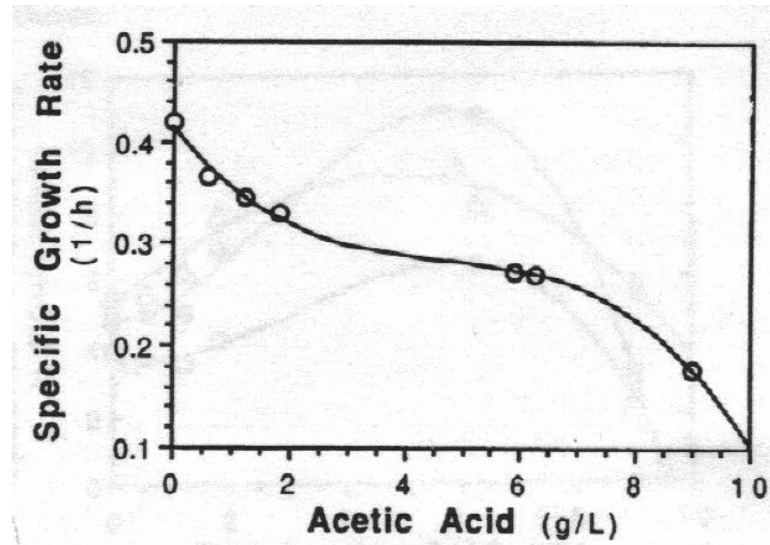


Fig. 3.2 Effect of acetic acid on the maximum specific growth rate of *Z. mobilis* [30]

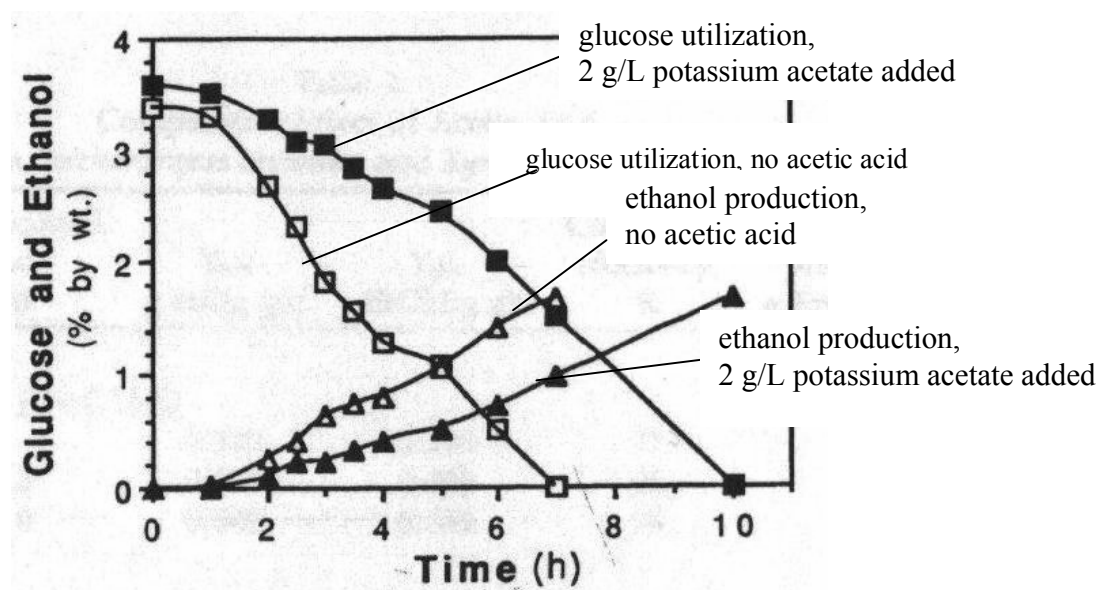


Fig. 3.3 Effect of acetic acid on the glucose metabolism by *Z. mobilis* at pH 5 [30]

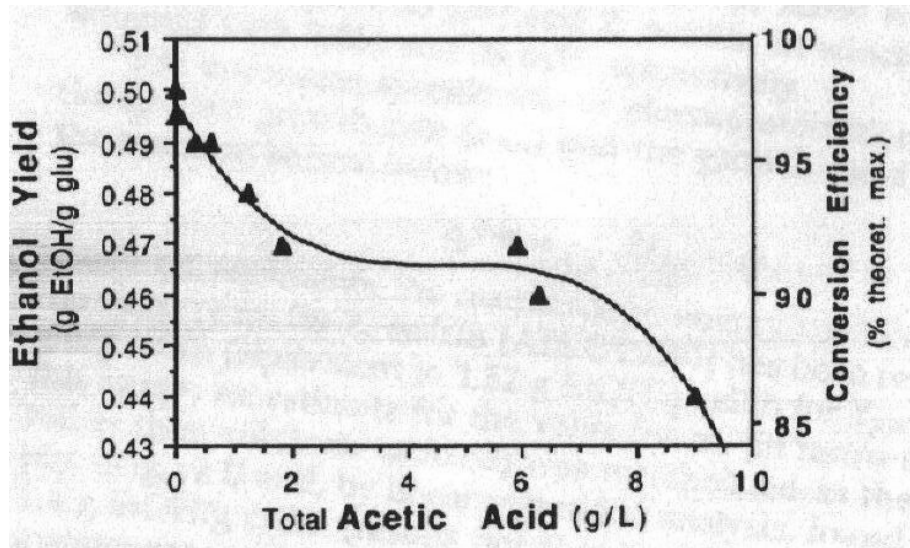


Fig. 3.4 Effect of acetic acid on ethanol yield [30]

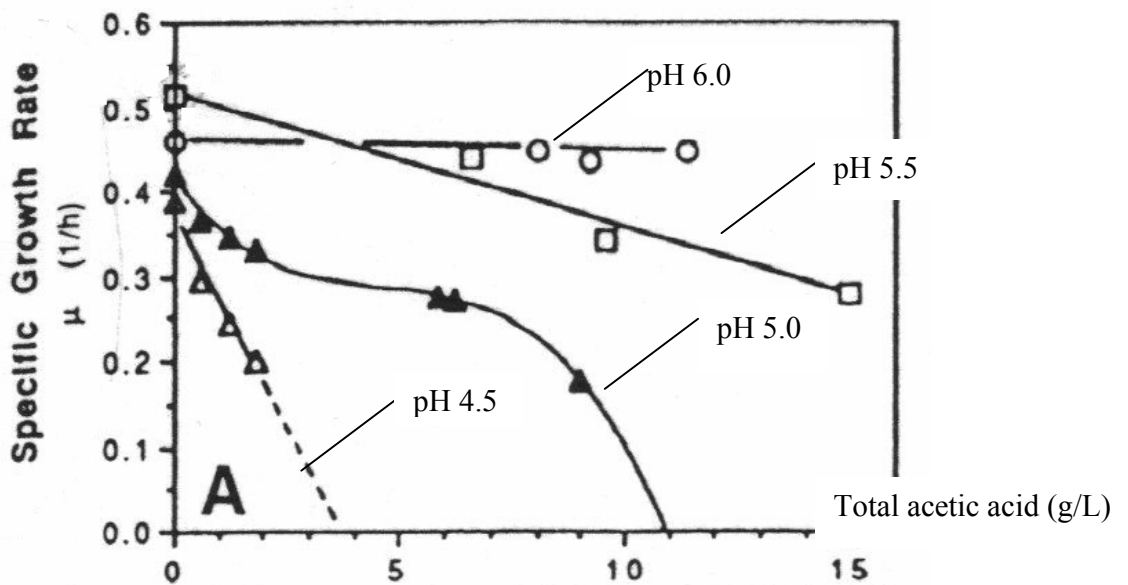


Fig. 3.5 Effect of pH and acetic acid on growth [67]

The effect of acetic acid is related to the pH. Lawford has carried out another series of experiments to study the effect of pH [67]. The growth of *Z. mobilis* at four different pH values over the range 4.5-6.0 was measured. The data illustrated in Fig. 3.5 shows that the specific growth rate decreases as pH decreases. At pH 4.5, the specific growth

rate is reduced by 50% at about 2 g/L acetic acid and complete inhibition is projected to be at 4 g/L. However concentrations of acetic acid greater than 10 g/L are tolerated if the pH is above 5.0. At pH 6.0, the specific growth rate with 12 g/L acetic acid is quite similar to that in the absence of acetic acid, although the growth yield is decreased by about 40%.

Figure 3.5 also shows the optimal pH shifts when acetic acid concentration increases. The optimum pH for growth is 5.5 in the absence of acetic acid. However the optimum pH shifts to 6.0 in the presence of 9 g/L acetic acid.

Kim studied the effect of acetic acid by ^{13}C NMR, which could monitor the progress of fermentation processes [33]. The addition of 10.9 g/L sodium acetate caused a decrease in both specific rates of xylose utilization and ethanol production at pH 5.5 and 6.0. And the addition of 10.9 g/L sodium acetate caused a complete inhibition of xylose utilization and ethanol production at a pH 5.0 or lower.

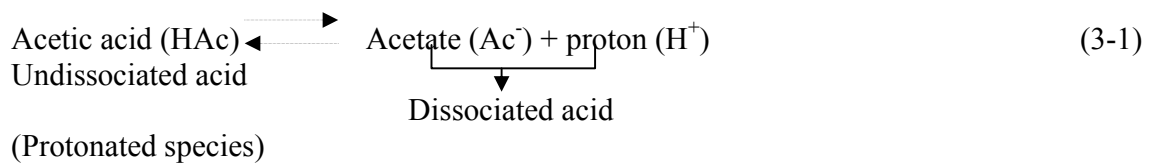
The inhibition of acetic acid is more significant when *Z. mobilis* ferments xylose than glucose. For example, Joachimsthal [32] measured the growth rate of *Z. mobilis* strain. The results showed that this *Z. mobilis* (ZM4) had the specific growth rate 0.32 h^{-1} in the presence of 5.87 g/L acetic acid and this number decreases to 0.21 h^{-1} in the presence of 8.78 g/L acetic acid. Jeon [31] measured the growth rate of *Z. mobilis* (ZM4), the same strain as Joachimsthal used, but carried pZB5 to enable this strain to ferment xylose. In the presence of 5.87 g/L acetic acid, the specific growth rate was 0.16 h^{-1} , and it reduced to 0.085 h^{-1} in the presence of 8.78 g/L. The specific growth rate of ZM4 (pZB5) was lower in the presence of the same amount of acetic acid than ZM4. And the reduction rate of the specific growth rate of ZM4 (pZB5) was also higher than that of ZM4.

The acetic acid also influences the growth of other microorganisms. For example, compared to the growth in the absence of acetic acid, at pH 7.0, the specific growth rate of anaerobic growth of *E.coli* K12(S) was reduced to 50% in the presence of only 0.7 g/L acetic acid and a complete inhibition occurred at 2.2 g/L acetic acid [67]. pH is also an important parameter. At pH 7.0, almost 60% of glucose was fermented to ethanol after twenty-four hours; however at pH 5.5, only 12% glucose was consumed after twenty-four hours, which accompanied a lower ethanol yield and slower fermentation. More severe inhibition with increasing acetic acid concentrations was also reflected in the reduction of cell growth yield. At pH 7.0, a cellular concentration of 0.31 mg/ml was reached after six hours of growth in the absence of sodium acetate. This concentration was reduced to 0.25 mg/ml in the presence of 12.0 g/L sodium acetate. The higher the sodium acetate concentration, the longer the lag phase was observed in comparison with the control medium without sodium acetate [67, 68].

Besides acetic acid, there are other inhibitors existing in the pretreated biomass. These inhibitors could be classified into three sorts: (1) furan derivatives, such as 2-furaldehyde and 2-furoic acid; (2) aliphatic acid, such as formic acid; (3) phenolic compounds, such as vanillin and hydroxybenzoic acid. Acetic acid belongs to the aliphatic acid.

3.2 Mechanism of acetic acid toxicity

Acetic acid (HAc) is a weak acid with a pKa of 4.75. It could be dissociated into anionic specie “acetate” (Ac^-) and a proton “(H^+)” by the following equation [30, 67, 68].



The ratio of “acetate” to acetic acid depends on pH. At a specified pH value, the relative concentrations of the dissociated and undissociated species are given by the Henderson-Hasselbalch equation:

$$\text{pH} = \text{pKa} + \log_{10}[\text{Ac}^-]/[\text{HAc}] \tag{3-2}$$

The relationship between pH and the concentration of undissociated acetic acid is displayed in Figure 3.6 (insert). At low pH, free acetic acid predominates, whereas at high pH, the ion form dominates. The concentration of the toxic species, HAc, decreases exponentially as the pH increases.

Two terms are needed to be cleared here. One is “acetic acid” and another is “acetate”. In this paper, the acetic acid content of the fermentation medium refers to the total mass of the acid, including both dissociated and undissociated forms.

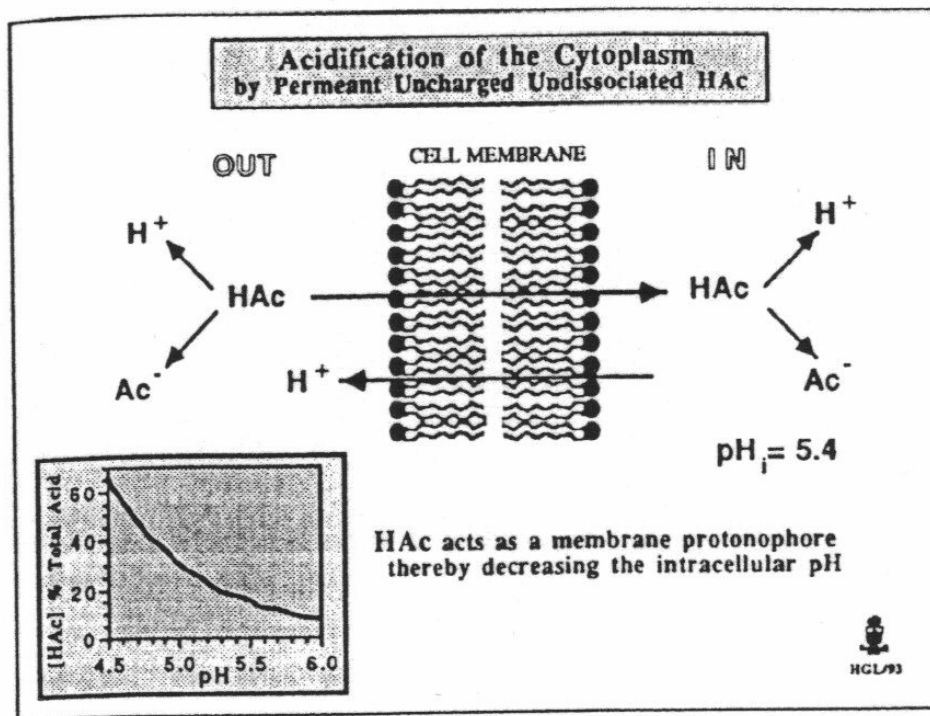


Fig. 3.6 Permeability of the cell plasma membrane to HAc – the undissociated form of acetic acid; and the concentration of HAc as a function of the pH of the medium (insert) [69]

The inhibition effect of acetic acid is dependent on the concentration of the undissociated species (HAc), because the uncharged form of low molecular weight weak acids, such as propionic and acetic acid, are soluble in the lipids of the cell membranes. Acetic acid could affect the growth of cells in two ways [30, 67, 69].

1) Acidification of the cytoplasm

Fig. 3.6 shows how the acetic acid acidifies the cytoplasm. The undissociated form of acetic acid (HAc) freely penetrates from outside of cell to inside. After the HAc penetrates, it would dissociate to ionic forms and generate protons, which lead to the reduction of pH value inside the cell. The reduced pH will affect many essential proteins

by protein denaturizing. The denatured proteins can have serious consequences, from loss of solubility to aggregation. All these changes may threaten the survival of cells [70]. The amount of undissociated HAc is related to pH. The lower the pH is, the more undissociated HAc will be available to transfer through the cell membrane (Figure 3.6, insert). The more released H^+ will accumulate in the cytoplasm, reducing cytoplasmic pH. In principle, the equilibrium distribution of acetic acid between the bulk phase of the culture medium and the cell cytoplasm could be calculated if the equilibrium dissociation constant (pK_a) is the same for these compartments by the following equations.

$$K = [H^+]_0[Ac^-]_0/[HAc]_0 = [H^+]_i[Ac^-]_i/[HAc]_i \quad (3-3)$$

At equilibrium,

$$[HAc]_0 = [HAc]_i \quad (3-4)$$

Therefore,

$$[Ac^-]_i/[Ac^-]_0 = [H^+]_0/[H^+]_i \quad (3-5)$$

And

$$\text{Log}_{10}\{[Ac^-]_i/[Ac^-]_0\} = \log[H^+]_0 - \log[H^+]_i = pH_i - pH_0 \quad (3-6)$$

The equilibrium concentration ratio $[Ac^-]_i/[Ac^-]_0$ is defined as the degree to which acetic acid is accumulated by the cell cytoplasm, and is called “accumulation factor” (AF), which can be determined as follows.

$$\text{Log}_{10}AF = pH_i - pH_0 \quad (3-7)$$

$$AF = \text{antilog} \Delta pH \quad (3-8)$$

Hence, acetic acid is distributed according to ΔpH . However the above equations are only valid at equilibrium and only if the amount of the undissociated acid is small

compared to the total amount of acid (i.e., when the pH_0 is at least 1 U higher than the pK_a or pH_0 is higher than 5.75).

2) Energetic uncoupling

“Coupled growth” (or “balanced growth”) means that the rate at which energy (ATP) required by the cell for growth is “balanced” by the rate of energy-yielding catabolism of the carbon source. Under certain conditions, the growth yield is lower than expected on the basis of ATP yield, this situation refers as growth and energy production are “uncoupled”. “Energetic uncoupling” occurs when energy is either wasted or used for maintenance (homeostatic) purposes rather than for growth [67]. Here energetic uncoupling occurs because the produced energy is used for maintaining the intracellular pH. The intracellular pH is maintained constant at about 5.4 [30, 69] by energy-linked membrane proton pumps which transport protons out of the cell and this process needs energy. In a batch culture, “energetic uncoupling” results in a decrease in growth yield independent of the rate of sugar utilization. In continuous fermentation, this effect could be represented by the maintenance metabolism. The maintenance energy coefficient is the amount of energy used for purposes other than growth. This number has been estimated to be 1.78, 1.53, 1.29 and 1.05 g ethanol/g biomass, at pH 4.5, 5.0, 5.5 and 6.0 respectively for *Z. mobilis* ZM4 [69]. The maintenance energy coefficient increases with decrease of pH, which support the conclusion that the effect of acetic acid is related to pH.

3) Anion accumulation

Another toxicity mechanism was also proposed, though not widely accepted [68]. It states that the acetic acid induces an anion accumulation, resulting in increased internal osmotic pressure of cells. To reduce the concentration of acetate inside the cell, cells may

induce a transport system, which requires energy. Consequently, growth yield decreases. No evidence was shown to support the proposed mechanism.

To understand the effect of acetic acid, Rogers and his co-workers studied the acetic acid inhibition of both *Z. mobilis* (ZM4) and recombinant ZM4 (pZB5) by ^{31}P nuclear magnetic resonance (NMR) [33]. Figure 3.7 shows the ^{31}P NMR spectra of ZM4 (pZB5) metabolizing glucose in the presence of 10.9 g/L of sodium acetate (A) in the absence of sodium acetate addition (B), at pH 5.5. Different levels of sugar phosphates and NTP formation were observed. The resonances of sugar phosphate and NTP were somewhat less than those of the control experiment, which means less biomass was produced than the control. The decrease in NTP could be the evidence of intracellular deenergization [71]. The shift of the sugar phosphate resonance toward the external phosphate resonance could probably indicate the pH downshift caused by the presence of acetic acid.

McMillan measured and analyzed the intracellular ATP levels in metabolically engineered *Z. mobilis* fermenting glucose and xylose mixtures [72]. Quantification of intracellular ATP levels was carried out using a TD-20/20 Luminometer, by measuring bioluminescence (BL) light intensity in relative light units (RLU), which was proportional to ATP concentration in the samples according to the BL luciferin-firefly luciferase reaction stoichiometry. The results showed that more ATP was consumed for growth, in other words, less ATP was required for cell maintenance, for the low acetic acid condition as compared to the medium with high acetic acid concentrations. This increased demand for ATP energy for non-growth-mediated cell maintenance during stressful conditions is consistent with uncoupling effect.

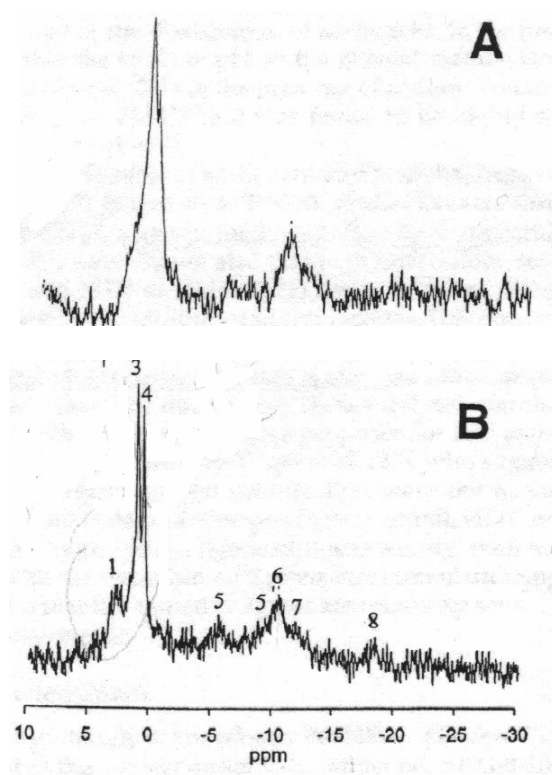


Fig. 3.7 Comparison of ^{31}P NMR spectra of *Z. mobilis* ZM4 (pZB5) at 2.5 min after addition of 277 mM glucose at 30°C and pH 5.5 in the presence (A) and absence (B) of 10.9 g/L of sodium acetate. 1, sugar phosphates; 3, extracellular phosphate; 4, triethyl phosphate as the internal standard; 5, NDP; 6, NAD and NADP; 7, UPD sugars; 8, β -NTP [33]

3.3 Tolerant mechanism

So far, no mechanism has been proposed for *Zymomonas mobilis* to explain how this organism responds to the acetic acid stress. However, there are some microorganisms that could naturally tolerate acetic acid up to 40 g/L. The best known of these are the so-called acetic acid bacteria (AAB). AAB is the commonly used term for the genera *Acetobacter* and *Gluconobacter*. Other less well – known species are at least as adept at growing and maintaining productivity under high acetic acid conditions. Studying these

naturally acetic acid tolerant strains could help to understand how the strains response the acetic acid stresses.

In 1990, Fukaya studied the acetic acid tolerant mechanism [73]. They developed five acetic acid tolerant strains from *Acetobacter* strain. After cloned the acetic acid resistance genes, they suggested that the acetic acid resistant gene(s) was located in this PstI fragment of plasmid pAR301. Their further study showed that three fragments A, B, and E were important for acetic acid tolerance and these fragments were designated as aarA, aarB, and aarC. They believed that cooperation of aarA, aarB, and aarC was necessary to confer acetic acid resistance to the host cell. Sequences of aarA and aarC have been published and on the basis of sequence homologies, it was concluded that aarA coded for a citrate synthase and aarC appeared to be involved in acetic acid uptake [74].

In 1997, Lasko studied the acetic acid tolerant mechanism by two-dimensional gel electrophoresis (2DE) [75]. The microorganisms were *A. aceti* and *G. suboxydans*. Proteins were expressed in the microorganisms grown in both normal medium and medium containing 10 g/L acetic acid. The results showed that many proteins exhibited altered expression levels in the stressed cells. After eliminating the proteins that were affected by both heat shock and acetic acid, only eight proteins left for each microorganism. These eight proteins, the characteristics of these proteins and their change are summarized in the Table 3.1.

Table 3.1 Relative concentrations of Asps in AAB during growth in the presence of 10 g/L acetic acid [75]

protein	MW (kD)	pI	<i>A. aceti</i>	<i>G. suboxydans</i>
AspA	42	5.9	Novel	Novel
AspB	26	5.6	+	Novel
AspC	18	6.3	Novel	Novel
AspD	34	5.5	+	
AspE	33	5.5	+	
AspF	21	4.7	++	
AspG	19	5.0	+	
AspH	19	5.5	++	
AspI	54	6.3		++
AspJ	53	5.5		++
AspK	49	5.9		++
AspL	37	6.5		Novel
AspM	37	6.7		Novel

Comparing the results found by Fukaya and Lasko, the proteins they found were not the same. aarA and aarC had a MW 48.9 kD and 53.5kD, respectively. The pI was 8.1 and 7.2 for aarA and aarC, respectively. Lasko proposed several possibilities to explain why the proteins they found were different from the proteins Fukaya found. (1) aarA and aarC were simply not expressed in these strains under their experimental conditions; (2) perhaps one or both are expressed, but at levels below the detection sensitivity of silver staining; (3) aarA and aarC might also be expressed and subsequently subjected to post

translational modifications and the pI and/or molecular weight was changed; (4) aarA and aarC might form complex structure with other cell debris and failed to enter the gel in either the first or second dimension. Although no conclusion can be reached which proteins are responsible for acetic acid tolerance, at least it can be sure that specific proteins are involved in acetic acid tolerance in AAB strains.

The gene expression of *Escherichia coli* was carried out by Arnold in 2001 by gene array [76]. Because the function of the genes inside the *E. coli* is best known, this study could help find the proteins responsible for acetic acid tolerance. The results showed that 26 genes in *E. coli* O157:H7 increased at least two fold after exposure to sodium acetate at pH 7 (Table 3.2). From this table, three kinds of proteins are identified. (1) Reducing the pH inside the cell. For example, well known genes - gadA and gadB encode isozymes of glutamate decarboxylase, which catalyzes the conversion of glutamate to γ -aminobutyrate. gadC is predicted to code for a γ -aminobutyrate antiporter. These three genes were proposed to function together to help maintain a near neutral intracellular pH when cells were exposed to extreme acidic conditions. (2) Synthesis of fatty acid in the cell membrane. The cfa gene codes for a cyclopropane fatty acid synthase. Cyclopropane fatty acid (CFA) is important for acid resistance. Based on the model proposed by Paula [77], proton permeability in lipid bilayers was inversely proportional to bilayer thickness. Conversion of unsaturated fatty acid (UFA) to CFA had the same effect as increased bilayer thickness. So CFA may help provide acid resistance by decreasing the permeability of the membrane to protons and produce acid resistance. (3) Reducing the osmotic pressure. osmC and osmY are related to this function. (4) Preventing periplasmic protein denatured by low pH. The genes hdeA and hdeB are related to the expression of

HdeA and HdeB, which are predicted to be periplasmic proteins. The expression of these two genes increases at low pH, so the function of protein could be maintained even some periplasmic proteins denaturized.

Table 3.2 *E.coli* genes whose relative expression level increases after treatment with sodium acetate [76]

Gene	Functional classification	Known or predicted function
Known RpoS-regulated genes		
AdhE	Metabolism (fermentation)	Aldehyde-alcohol dehydrogenase
cfa	Metabolism	Cyclopropane fatty acyl phospholipids synase
dps	Adaptation (starvation)	DNA-binding stress-induced protein
gadA	Metabolism	Glutamate decarboxylase-alpha
gadB	Metabolism	Glutamate decarboxylase-beta
gadC (xasA)	Transport	Predicted amino acid antiporter
hdeA	Not known	Protein regulated by H-NS, chaperone
hdeB	Not known	Protein regulated by H-NS, predicted chaperone
katE	Protection (oxidative stress)	Catalase (hydroperoxidase HP11)
osmC	Adaptation (osmotic pressure)	Osmotically inducible protein C
osmY	Adaptation (osmotic pressure)	Osmotically inducible periplasmic protein

Table 3.2 <i>E.coli</i> genes whose relative expression level increases after treatment with sodium acetate (continue)		
Other genes		
aslB	Metabolism; chaperoning	Putative regulator of arylsulfatase (AslA) activity
fbaB (dhnA)	Metabolism (glycolysis)	Class I fructose-bisphosphate aldolase
glnK	Metabolism (nitrogen metabolism)	Activator of NRII (GlnL/NtrB) phosphatase
grxB	Metabolism	Glutaredoxin 2
hdeD	None	Protein regulated by H-NS
metA	Metabolism	Homoserine transsuccinylase
ompC	Transport	Outer membrane porin OmpC
pflB	Metabolism	Pyruvate formate lyase I
slp	Adaptation (starvation)	Outer membrane lipoprotein
talA	Metabolism	Transaldolase A
yccJ	None	Hypothetical protein
yeaQ	None	Hypothetical protein
yhbH	Information transfer	Predicted sigma-54 modulation protein
yhiW	Information transfer	Predicted AraC-type regulatory protein
yhiX	Information transfer	Predicted AraC-type regulatory protein

On the other hand, the expression of sixty genes decreased by at least 50% after the addition of sodium acetate. Some of these genes encode components of the transcription – translation machinery. However these changes may be due to the decreased growth rate caused by addition of sodium acetate. The depression of these genes may not be the response of strains caused by sodium acetate.

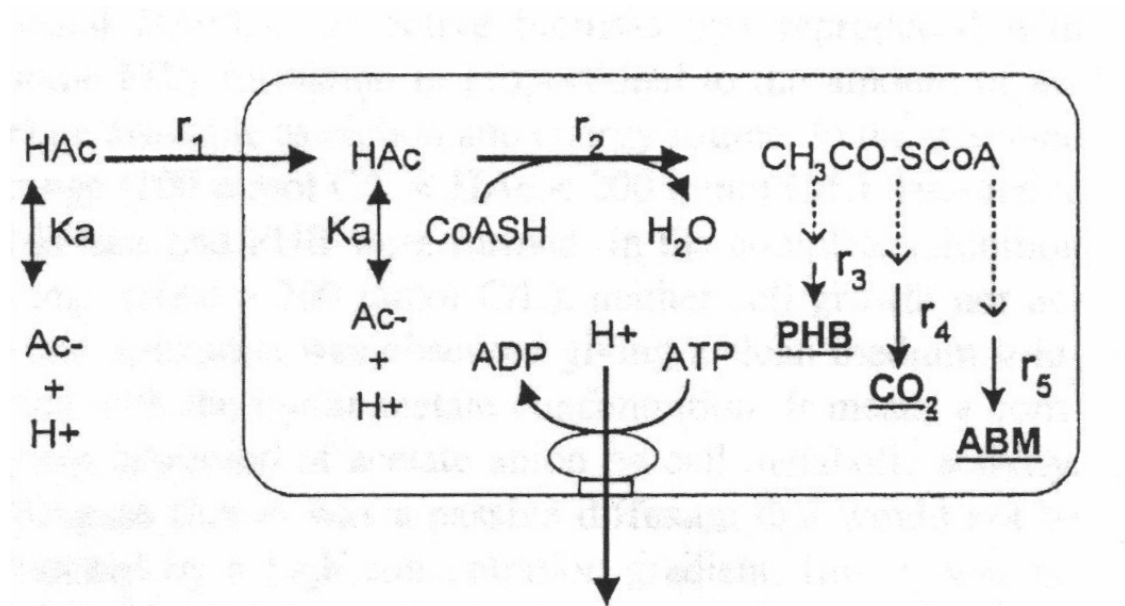


Fig. 3.8 A cell model with five fluxes in utilization and detoxification of acetic acid by *R. eutropha* [78]

Another possible mechanism to increase the resistance of acetic acid of microorganisms is to consume the acetic acid by TCA cycle. Yu studied the detoxification of acetic acid by *Ralstonia eutropha* [78]. This strain could consume acetic acid by TCA cycle. They proposed a metabolic flux model and it is illustrated in Figure 3.9. In this model, the acetic acid went through the TCA cycle and appeared in three metabolic products, PHB, CO₂ and active biomass. Though this strain is unique because it could grow on acetic acid as its sole carbon and energy source, it gives another

possibility that when a microorganism grows in acetic acid environment, it induces genes that could help to digest the acetic acid inside the cell.

In summary, acetic acid could affect organisms by two mechanisms: (1) acidification of the cytoplasm and (2) “energetic uncoupling”. The effect of acetic acid is related to the concentration of concentration and pH. The microorganisms may increase the resistance to acetic acid by the following mechanisms: (1) maintaining the intracellular pH; (2) changing the fatty acid of cell membrane; (3) protecting the protein from denaturizing and (4) consuming acetic acid by TCA cycle.

CHAPTER 4 EXPERIMENTS

4.1 Microorganism and culture maintenance

Wild type *Zymomonas mobilis* ZM4 (ATCC31821) was obtained from ATCC (American Type Culture Collection). Mutants were developed from the wild type strain by adaptive mutation. Among these mutants, ZM6010, ZM6012, ZM6014, ZM5510 and ZM5010 were chosen for more careful characterization. Mutants ZM6010, ZM6012, ZM6014 were obtained by adaptation at high acetic acid concentration. Mutants ZM5510 and ZM5010 were obtained by adaptation at low pH. Mutants ZMNTG5514, ZMNTG5516, ZMNTG6014 and ZMNTG6014 were developed by NTG mutagenesis from ZM5510.

For long-term storage, all strains were kept at -80°C in 30% (w/v) glycerol solution by mixing 500µl sterile medium with culture (overnight cultured) with 500µl 60% (w/v) glycerol solution in a 1ml vial. Glycerol solution was prepared by mixing glycerol and deionized water. The 60% glycerol solution was autoclaved at 120°C for 20 minutes.

4.2 Medium

Different mediums were needed for different experiments. Seed medium was used for adaptive mutation. Solid medium was used for single colony screening. And fermentation medium was needed for mutant characterization.

4.2.1 Seed medium

Seed medium contained 20 g/L glucose, 10 g/L yeast extract, 2 g/L monobasic potassium phosphate. This medium was known as RM medium. Acetic acid (0.2% to 1.6% w/v) was added when necessary. Sodium hydroxide (50% w/v) was used to adjust the pH of liquid medium. This medium was then sterilized by filtration using a 0.22 μm filter.

4.2.2 Fermentation medium

Fermentation medium was RM medium based with increased glucose concentration to 50 g/L. Acetic acid concentration varied from 0.2% - 1.6%. pH of medium was adjusted by sodium hydroxide (50% w/v). The medium was sterilized by filtration using a 0.22 μm filter.

4.2.3 Medium for other inhibitors experiments

The same fermentation medium was used except inhibitors were added at different concentrations: vanillin (0.5 g/l, 1 g/l), formic acid (2.68 g/l, 5.37 g/l), hydroxybenzoic acid (3.4 g/l, 6.8 g/l) and furfuryl alcohol (3.89 g/l, 7.7 g/l). The pH of medium was adjusted to 6.0 by sodium hydroxide (50% w/v). The mediums were sterilized by filtration using a 0.22 μm filter.

4.2.4 Solid medium for agar plates

The solid medium was a mixture of 100 ml autoclaved RM medium containing 1.5% agar and 100 ml sterilized RM medium with various amount of acetic acid and sodium hydroxide (50% w/v) for pH adjustment. This mixture was then spread on the agar plate. Each agar plate had around 25 ml liquid medium.

4.3 NTG mutagenesis

Mutant ZM5510 was obtained through adaptation at pH 5.5. This strain was treated by NTG (N-methyl N'-nitro N-nitrosoguanidine). ZM5510 was firstly grown in the RM-acetate medium in the presence of 1.0% acetic acid at pH 5.5 and 30°C. There was no shaking in this process. After the O.D. value of the culture reached 0.6, NTG was added to the culture to a final concentration of 40 mg/l. After one hour incubation at 30°C without shaking, the culture was diluted to O.D. 0.08 and plated onto agar plates, prepared with different pHs (5.0, 5.5, and 6.0) and acetic acid concentrations (1.2%, 1.4%, and 1.6%). 100 µl culture was used for each plate. For each condition, cultures were spread onto six plates.

4.4 Colony screening

After plating, single colonies were formed on the agar plates after two days. These single colonies had different sizes (Figure 4.1). However the difference in size became smaller with increased acetic acid concentration or decreased pH. The number of single colonies on agar plate also decreased with increased acetic acid concentration or decreased pH.

Single colonies with larger size, seen from the naked eyes, were inoculated to the fermentation medium. 10 ml culture tubes were used to carry out the fermentation. All culture tubes were put in the incubator and the cells were incubated at 30°C without shaking. After the O.D. value of culture researched 0.1, samples (1 ml) were picked every two hours for O.D. measurement. After one day fermentation, 1 ml sample was taken for ethanol measurement.



Fig. 4.1 Agar plate for colony screening

4.5 Mutant characterization

The growth of the strains was studied by batch fermentations. The strains were grown in the fermentation medium with three different pHs (5.0, 5.5, and 6.0) and several acetic acid concentrations (0.0%, 1.0%, 1.4%, and 1.6%). 20 ml glass vials were used to carry out the fermentations. The glass vials were filled fully with medium to form an anaerobic cultivation. The glass vials were placed in a biological incubator. The

temperature was kept at 30 °C. There was no shaking in this process. The initial O.D. value was always 0.01. 1 ml sample was picked every four hours for both O.D. measurement and ethanol, glucose measurement.

4.6 Analytical methods

4.6.1 Optical density (O.D.)

O.D. value of the sample was measured by Beckman spectrophotometer DU530 at 600 nm. Specific growth rate was determined from the plots of optical density (OD) vs. time. The behavior of cells in exponential growing phase as a function of time could be described with the following equations [79]:

$$Y = ae^{\mu t} \quad (4-1)$$

a is the original O.D. value 0.01.

μ is defined as the specific growth rate (h^{-1}).

Y is the O.D. value and t is time (h).

$$\text{So } \ln(Y/0.01) = \mu t \quad (4-2)$$

When $\ln(Y/0.01)$ is plotted with growth time, a linear line should result, and the slope is μ .

The following example illustrates the calculation of μ , the specific growth rate. O.D. measurement was tabulated in Table 4.1, along with the corresponding natural log value. As shown in Figure 4.2, the natural logarithm of O.D. value was then plotted as a function of time. The first six points corresponds cells in exponential growing phase. These six points were used to calculate μ by linear regression. In this example, the slope is found to 0.27. Therefore, μ , in this case, is 0.27 h^{-1} .

Table 4.1 O.D. values and its natural logarithm value at different times

Time (hours)	0	4.10	7.78	9.23	12.32	15.18	21.60	47.77
O.D.value	0.01	0.026	0.066	0.102	0.266	0.548	0.907	0.891
LN (O.D.value/0.01)	0	0.956	1.887	2.322	3.281	4.004	4.508	4.490

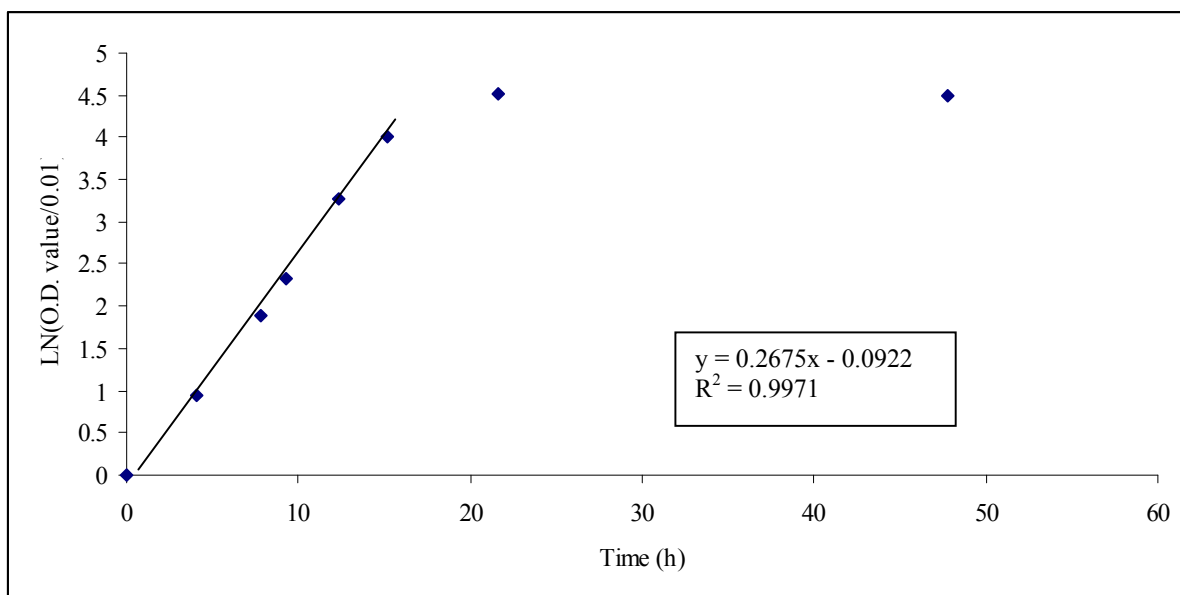


Fig. 4.2 Natural logarithm of O.D. value as a function of time

4.6.2 Ethanol measurement

Ethanol standards were prepared by mixing 1 ml HPLC grade water with various amounts of 100% ethanol (HPLC grade). The amounts of 100% ethanol were 0, 6, 13, 20, 26, 32, 40, and 47 μ l, respectively. The corresponding ethanol concentrations were 0, 0.47, 1.01, 1.54, 2.00, 2.45, 3.03, and 3.54 % (w/v), respectively. 100 μ l standard solution and 300 μ l 10% TCA (trichloroacetic acid) were mixed by vortexing. 200 μ l were

transferred to the HPLC vial for HPLC analysis. Ethanol was measured by Agilent 1100 HPLC equipped with an RI monitor and computer-interfaced controller. Separations were performed in a supelcogel H column. The injection volume was 10 μ l. The calibration line was then plotted. Calibration line is a relationship between ethanol concentration and the area (nRIU*s). A typical calibration curve is shown in Figure 4.3.

Samples from fermentation medium were prepared as follows. After O.D. measurement, 1 ml sample was centrifuged by a microcentrifuge for 10 minutes at 13000 rpm. Supernatants were collected. 100 μ l supernatants were mixed with 300 μ l 10% TCA in a vial. The mixture was then put on the ice for 10 minutes, followed by centrifugation for 10 minutes at 13000 rpm. 200 μ l supernatants were transferred to a HPLC vial for analysis. Area (nRIU*s) of each sample was obtained. Ethanol concentration was then calculated from the calibration line.

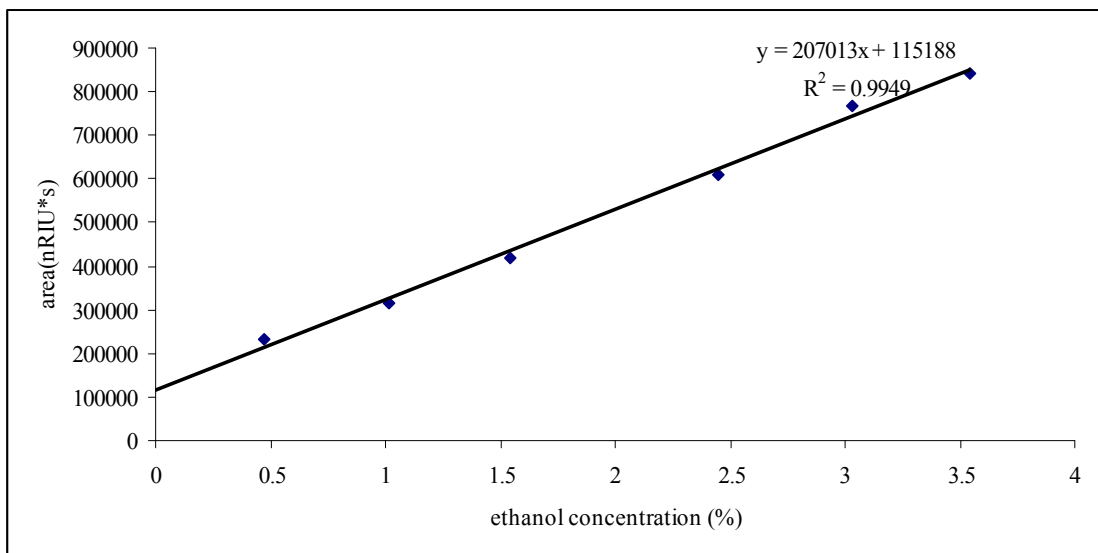


Fig. 4.3 Ethanol calibration line

4.6.3 Glucose and acetic acid measurement

HPLC analysis allows simultaneous measurement for ethanol, glucose, and acetic acid. Calibration lines for glucose and acetic acid were prepared with RM medium. Examples of calibration lines are shown in Figure 4.4 and 4.5. Concentrations of glucose or acetic acid in fermentation samples were calculated from calibration curves.

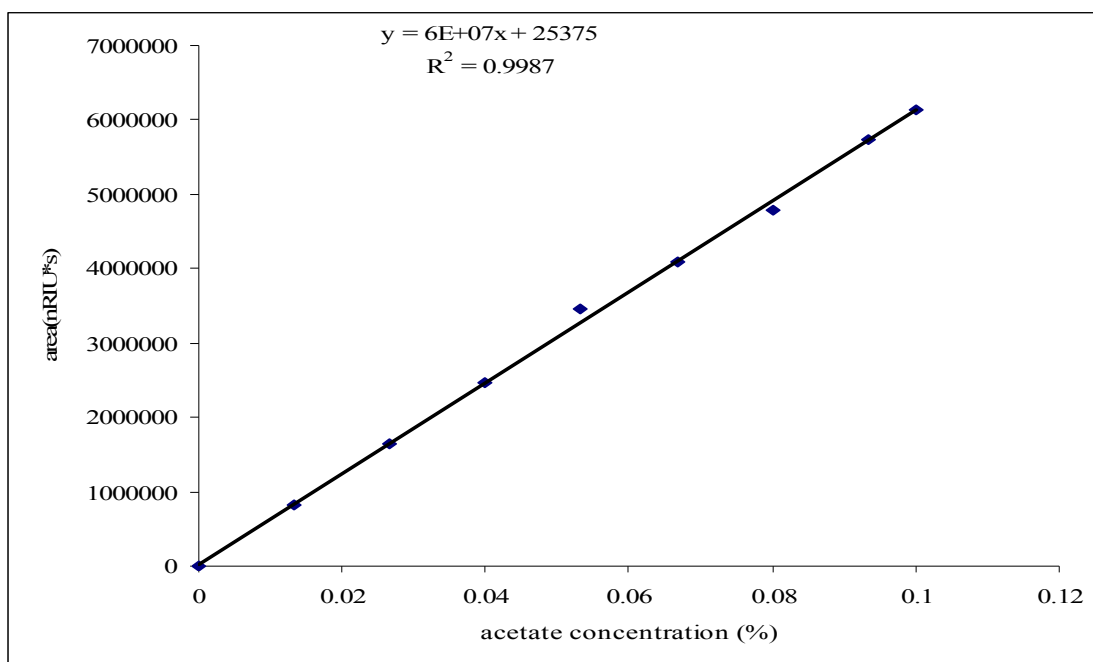


Fig. 4.4 Acetate calibration line

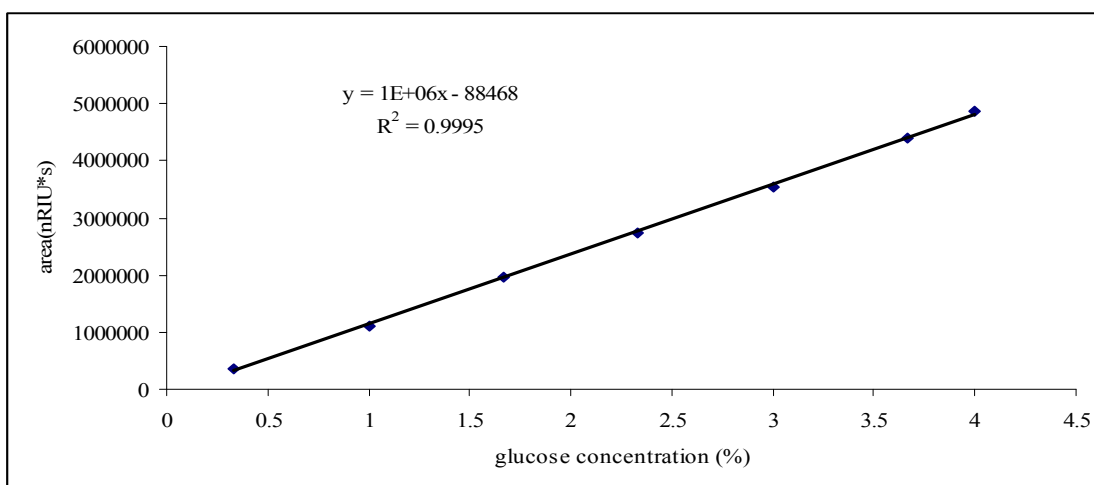


Fig. 4.5 glucose calibration line

CHAPTER 5 ADAPTIVE MUTATION

5.1 Adaptive mutation and mutant selection

Adaptive mutation is the method used in this research to develop acetic acid tolerant strains.

The procedure of adaptive mutation is illustrated in the Figure 5.1, using the first round adaptation as an example. In the first step, *Z. mobilis* (ZM4) strain was grown in RM medium in the absence of acetic acid at pH 6.0 and 30°C without shaking. After overnight growth, the *Z. mobilis* was inoculated into a new culture tube containing RM-acetate medium supplemented with acetic acid concentration (0.05%, 0.1%, 0.15%, and 0.2%). The initial O.D. value was 0.01. The culture was then incubated under the same condition as above until the O.D. value reached 0.1. An aliquot of culture was inoculated into a new culture tube containing the same but fresh RM-acetate medium to an O.D. of 0.01, and the cells were allowed to grow. Once O.D. reached 0.1, a new culture was started. The cycle repeated several times until the O.D. of the twenty-four hour culture reached a constant. The culture from the last cycle was then plated on an agar plate containing the same concentration of acetic acid as the liquid medium. Single colonies on the agar plates were screened based upon three parameters. These are: the specific growth rate, biomass concentration (24 hours) and ethanol concentration (24 hours). A mutant adapted to the highest acetic acid concentration of the round (in this case, 0.2%) was then used as the parent strain in the next round adaptation with an increment increase of acetic acid concentration.

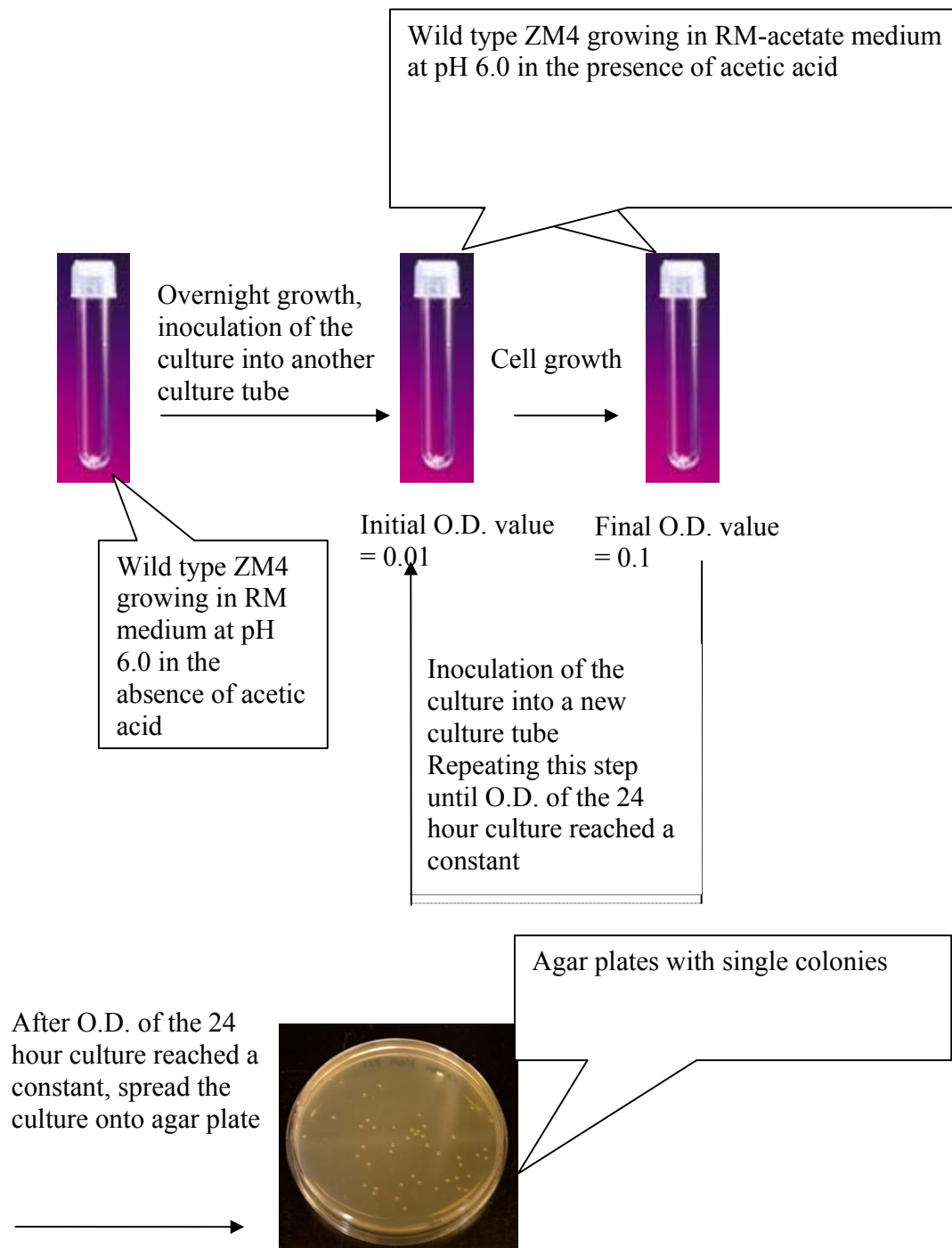


Fig. 5.1 Adaptive mutation process

Several rounds of adaptation mutation were run, generating several useful tolerant strains. Besides using acetic acid concentration as selective pressure, adaptation was also carried out at lower pH. Finally, NTG mutagenesis was also performed for selected mutant. The interrelations of the mutants obtained from this work are shown in Figure 5.2.

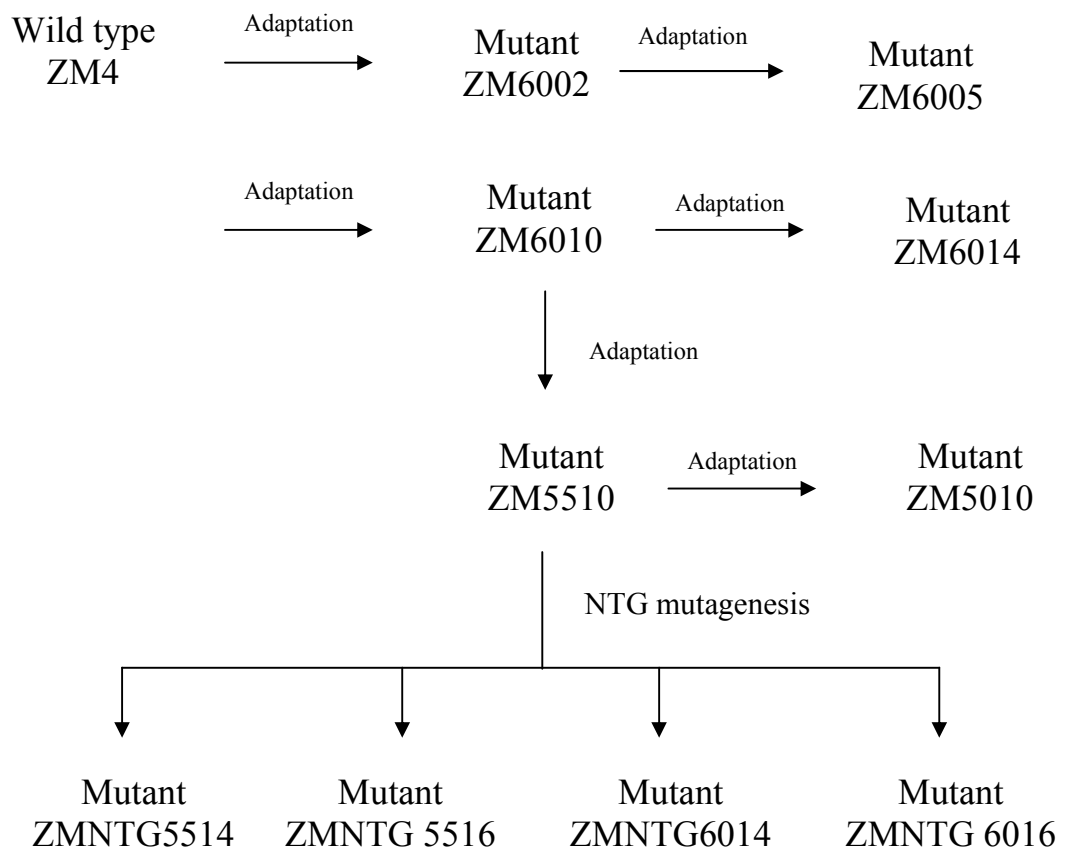


Fig. 5.2 The sequence of mutation

Note that the mutants obtained are named using a combination of letters and numbers. The letters “ZM” are taken from the name of the microorganism “*Zymomonas mobilis*”. The first two numbers following “ZM” denote the pH used in the adaptation and the last two numbers indicate the acetic acid concentration in percentage. For

example, ZM6002 is a strain obtained using adaptation conditions, pH 6.0, and 0.2% acetic acid concentration.

5.1.1 Adaptive mutation by gradually increasing the acetic acid concentration

The first adaptation experiment was carried out with acetic acid concentration, 0.05%, 0.1%, 0.15%, and 0.2%, respectively. At the end of the process (as described in previous section), the culture with the highest acetic acid concentration (in this case, 0.2%) was plated on agar plates containing RM medium (pH = 6.0) and 0.2% acetic acid. Twelve biggest colonies were selected for further study.

The selected twelve colonies were screened based upon, growth rate, cell mass concentration (O.D. obtained after 24 hour cultivation), and ethanol concentration (5% glucose and 24 hours cultivation).

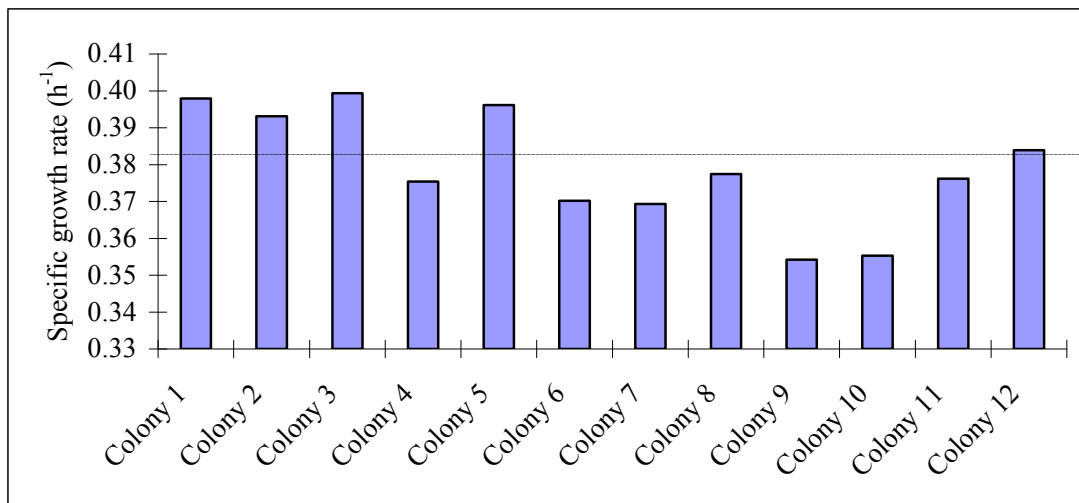
Figure 5.3 shows the specific growth rate, the O.D. and ethanol concentration for each colony. The horizontal lines indicate the average value of the 12 colonies. For specific growth rate, the average value was 0.38h^{-1} . The data showed that colonies 1, 2, 3, 5 had slightly higher specific growth rate than the average.

Biomass concentration (as indicated by O.D. after 24 hours cultivation) of colonies 1, 2, 3, 5 were all in the narrow range of 1.21 to 1.44, so further information was needed to pick the best single colony.

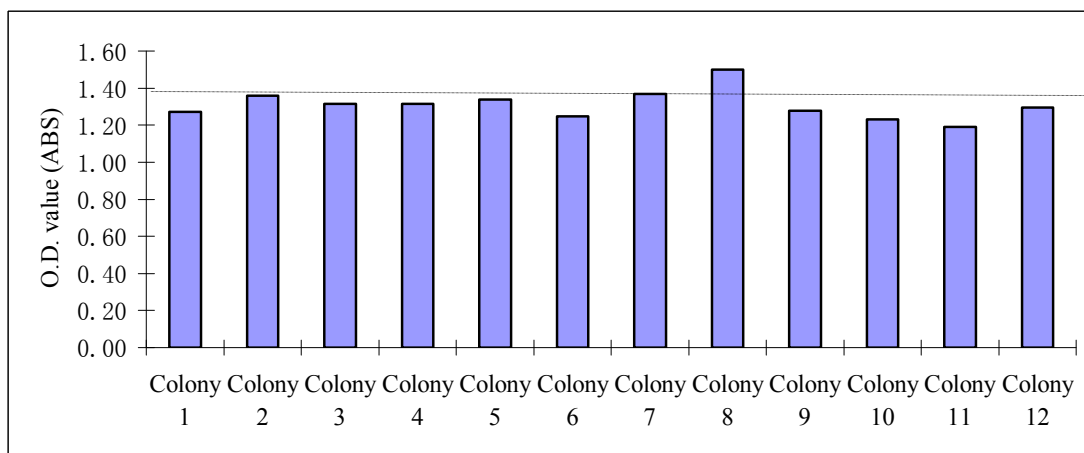
Ethanol concentrations of twelve different colonies after one-day fermentation showed that colony 1 and colony 2 had higher ethanol concentration than other colonies. The glycerol stocks were made for both colonies and stored in the -80°C freezer. Because both the O.D. value and ethanol concentration were higher for colony 2, compared to

colony 1. Colony 2 was picked for further adaptive mutation. This mutant was named ZM6002.

(A)



(B)



(C)

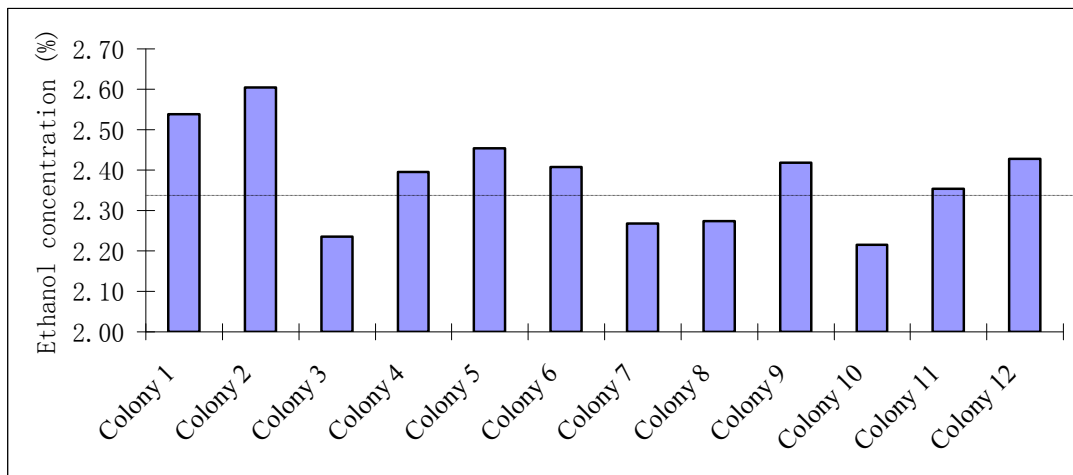


Fig. 5.3 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of twelve colonies from adaptive mutation with 0.2% acetic acid (Horizontal lines represent the average of the twelve colonies analyzed.)

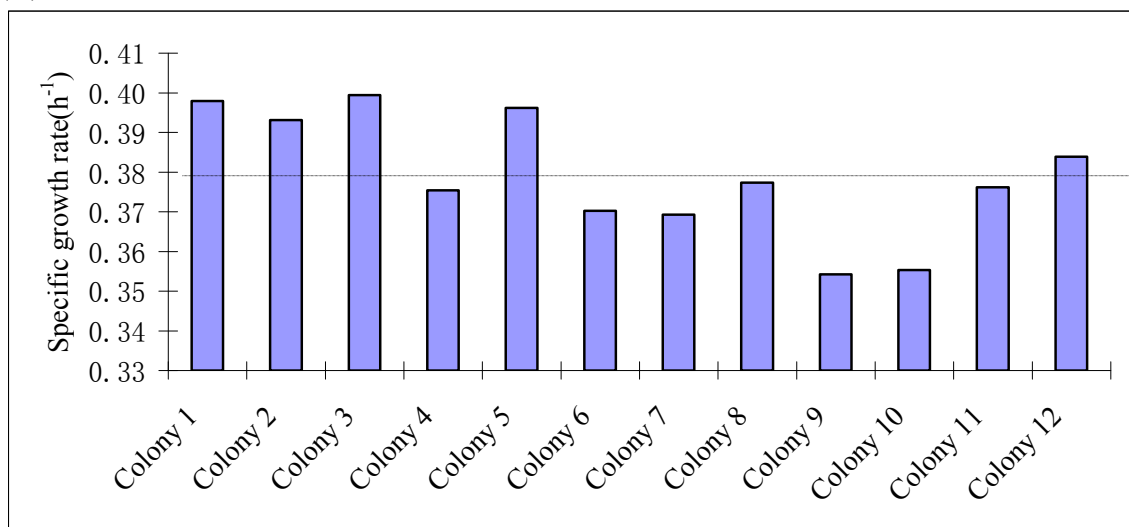
In the next round, with ZM6002 as parental strain, higher acetic acid concentrations of 0.3%, 0.4%, and 0.5% were used. The increment was increased from 0.05% to 0.1%. The same adaptation procedures were followed as the previous round. At the end of the adaptation, cultures were plated on agar plates containing the respective concentration of acetic acid.

Numerous colonies appeared on the agar plate containing 0.5% acetic acid, indicating successful adaptation to this acetic acid concentration. Twelve biggest colonies were selected from the agar plate for screening based upon, growth rate, cell mass concentration (O.D. obtained after 24 hour cultivation), and ethanol concentration (5% glucose and 24 hours cultivation).

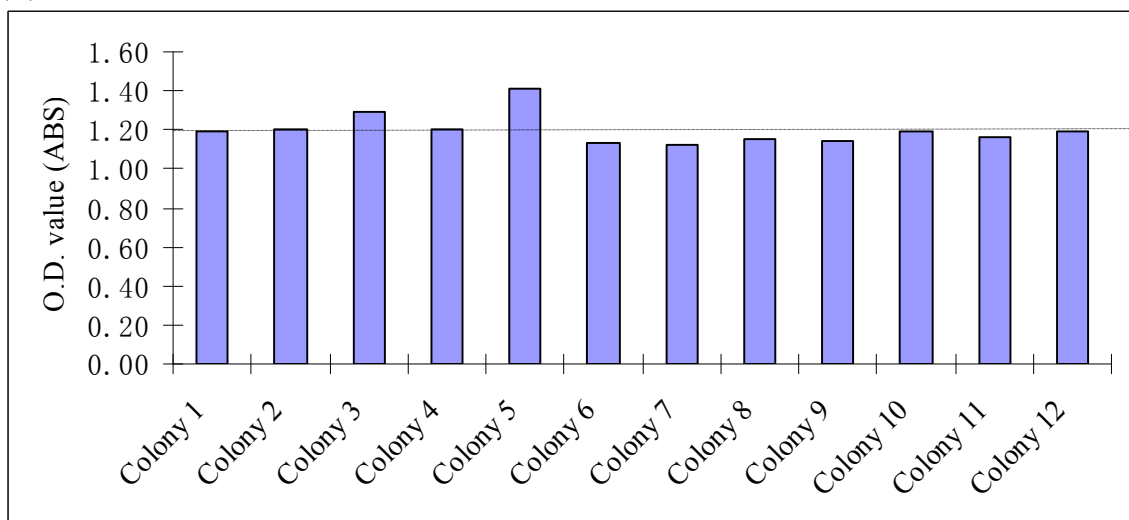
Figure 5.4 shows the specific growth rate, O.D. and ethanol concentration of each colony. For specific growth rate, the average value was 0.38h^{-1} . The data showed that colonies 1, 3, 5 had slightly higher specific growth rates than average.

However the ethanol concentration of colony 5 was lower than colony 1 and 3. And the biomass concentration of colony 3 was a slightly higher than colony 1. So colony 3 was selected for further adaptive mutation, this mutant was named ZM6005. The glycerol stocks were made for this mutant and stored in a -80°C freezer.

(A)



(B)



(C)

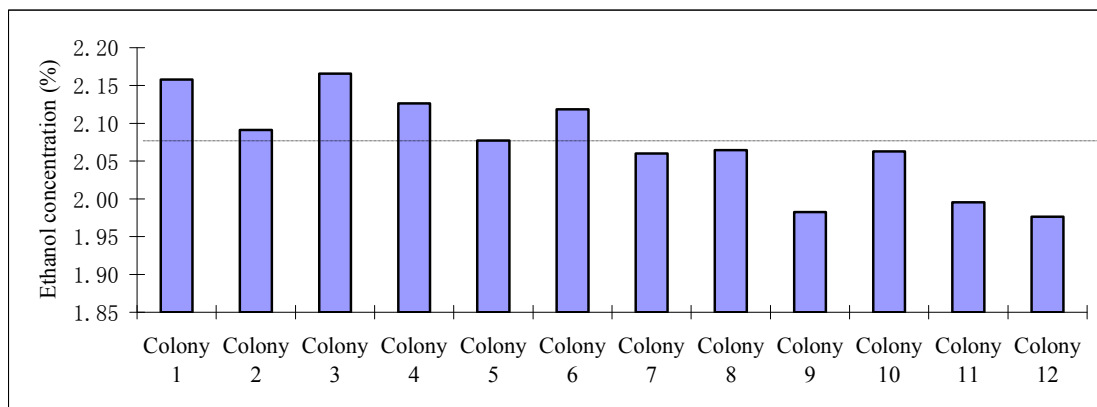
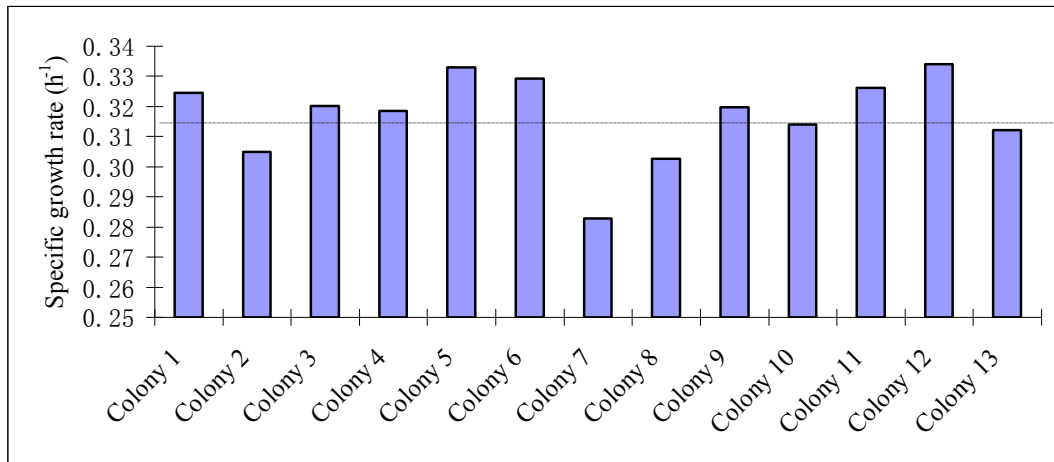


Fig. 5.4 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of twelve colonies from adaptive mutation with 0.5% acetic acid (Horizontal lines represent the average of the twelve colonies analyzed.)

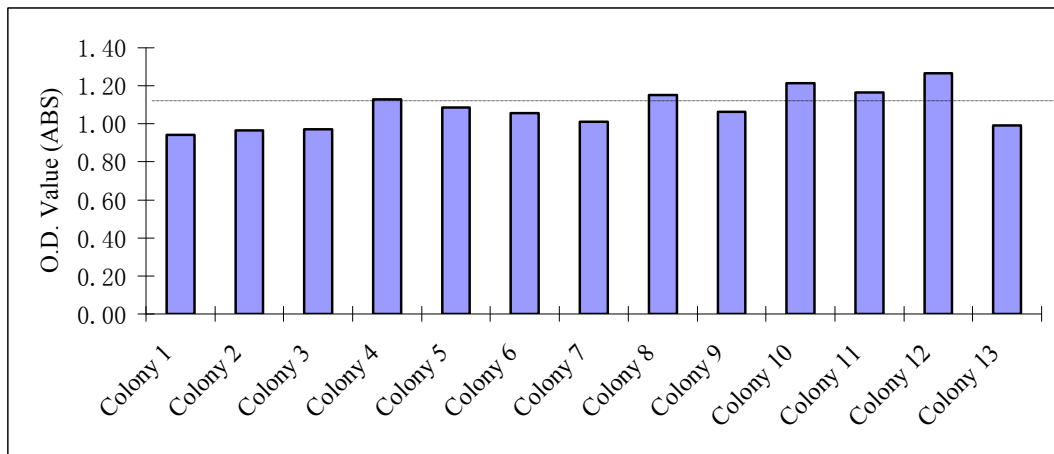
In the third round, mutant ZM6005 was adapted in the RM-acetate medium with 0.6%, 0.7%, 0.8%, 0.9% and 1.0% acetic acid. The increment of 0.1% was kept because this worked well in the last round. The same adaptation procedures were followed. After plating the culture on agar plate with respective acetic acid concentration, single colonies were formed on the agar plate containing 1.0% acetic acid, indicating successful adaptation to 1.0% acetic acid. Thirteen biggest colonies were selected from the agar plate (1.0% acetic acid) for screening based upon, growth rate, cell mass concentration (O.D. obtained after 24 hour cultivation), and ethanol concentration (5% glucose and 24 hours cultivation).

Figure 5.5 shows the specific growth rate, O.D. and ethanol concentration of each colony. Based on these three parameters, colony 5 was picked for further adaptive mutation. This mutant was named ZM6010. The glycerol stocks were made for this colony and stored in the -80°C freezer.

(A)



(B)



(C)

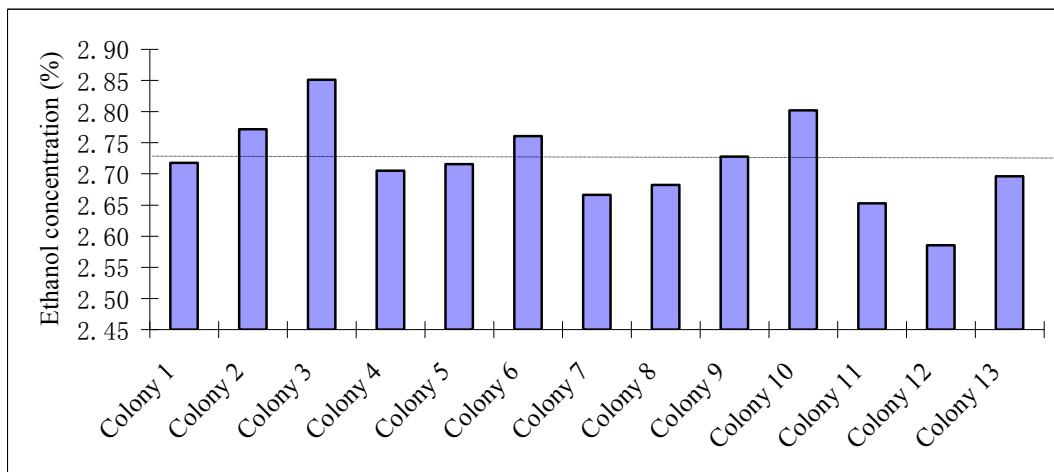
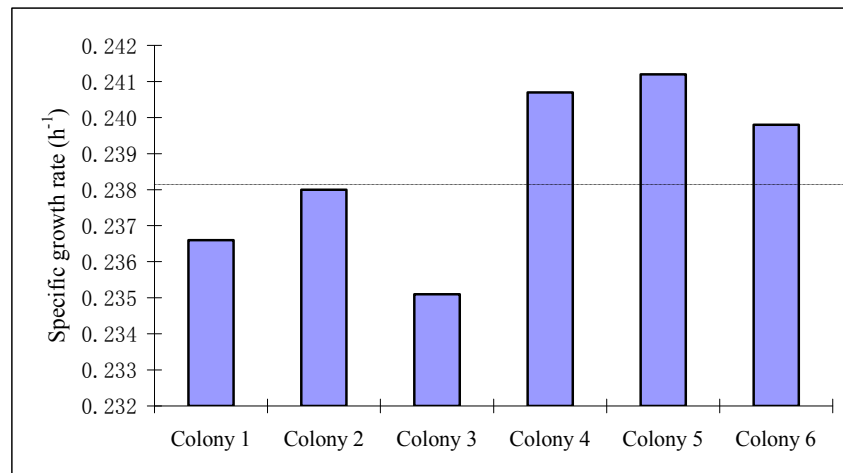


Fig. 5.5 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of thirteen colonies from adaptive mutation with 1.0% acetic acid (Horizontal lines represent the average of thirteen colonies analyzed.)

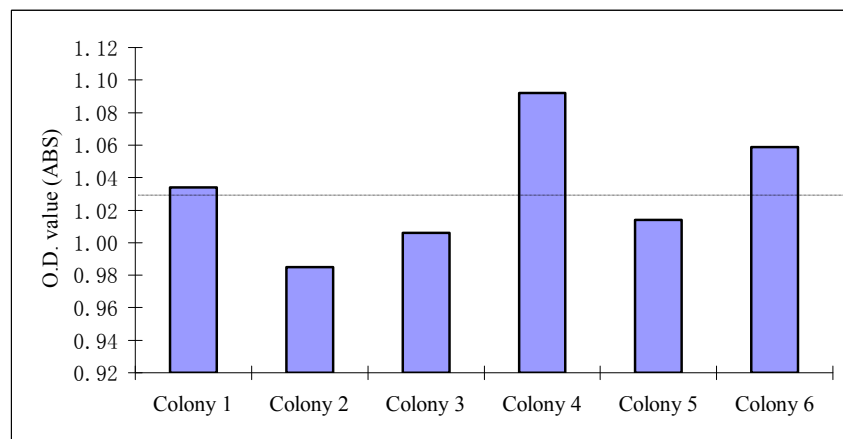
In the fourth round, mutant ZM6010 was adapted to acetic acid concentration 1.2%, 1.4% and 1.6%, respectively. The increment of 0.2% acetic acid concentration was used. Other conditions for adaptation remained unchanged. After plating, single colonies were formed only on agar plates containing 1.2% and 1.4% acetic acid, indicating successful adaptation to 1.2% and 1.4% acetic acid concentration, but not for 1.6% acetic acid. Single colonies from plates containing 1.2% and 1.4% were screened, because they appeared to tolerate higher acetic acid concentration than reported ZM4 in the literature, which was only 1.17%. As the acetic acid increased, adapted strains became more difficult to grow on the agar plate. There were only six big colonies on the plates containing 1.2% and 1.4% acetic acid. These colonies were subjected to the same screening procedure as the previous rounds.

Figure 5.6 shows the screening results of the six colonies selected from plate containing 1.2% acetic acid. Based on the three parameters (specific growth rate, O.D. and ethanol concentration), colony 4 was selected for further study. This mutant was named ZM6012. The glycerol stocks were made for this strain and stored in the -80°C freezer.

(A)



(B)



(C)

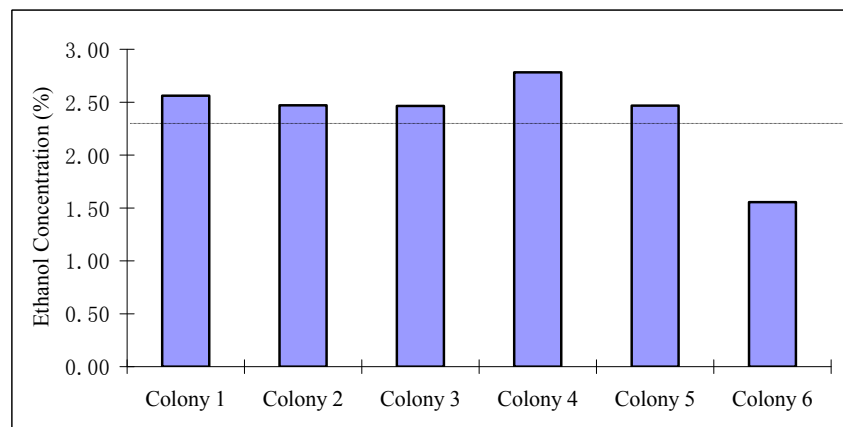
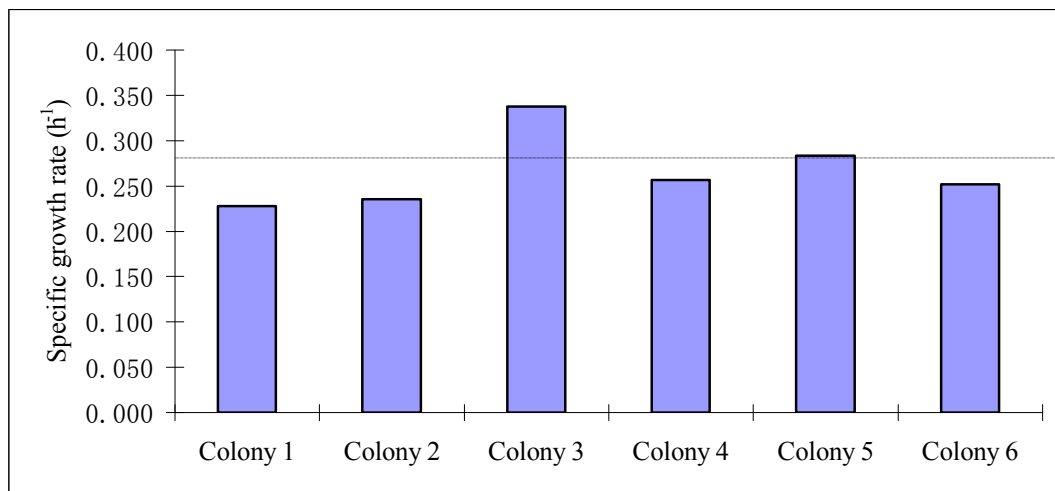


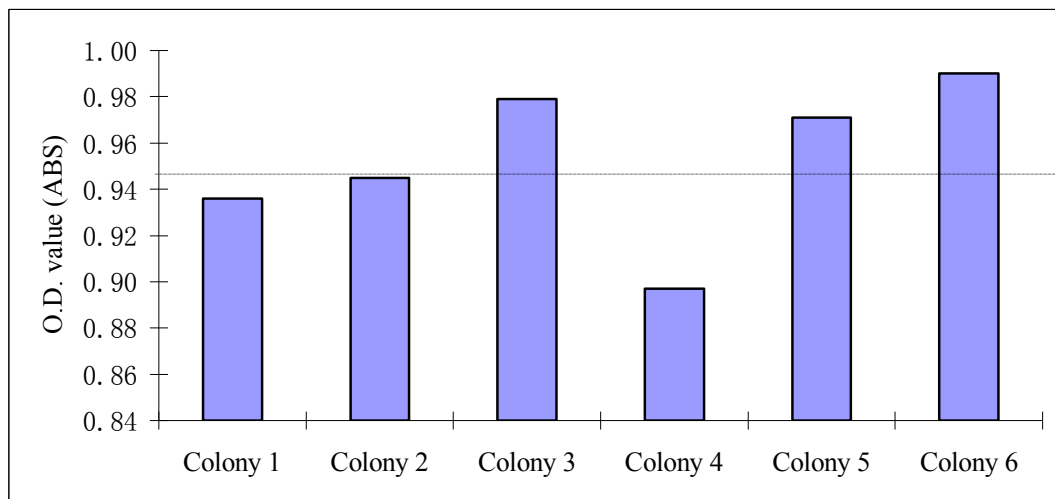
Fig. 5.6 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of six colonies from adaptive mutation with 1.2% acetic acid (Horizontal lines represent the average of six colonies analyzed.)

Figure 5.7 shows the screening results of the six colonies selected from plate containing 1.4% acetic acid. Based on the screening results, colony 3 was picked for further study. This mutant was named ZM6014. The glycerol stocks were made for this strain and stored in the -80°C freezer.

(A)



(B)



(C)

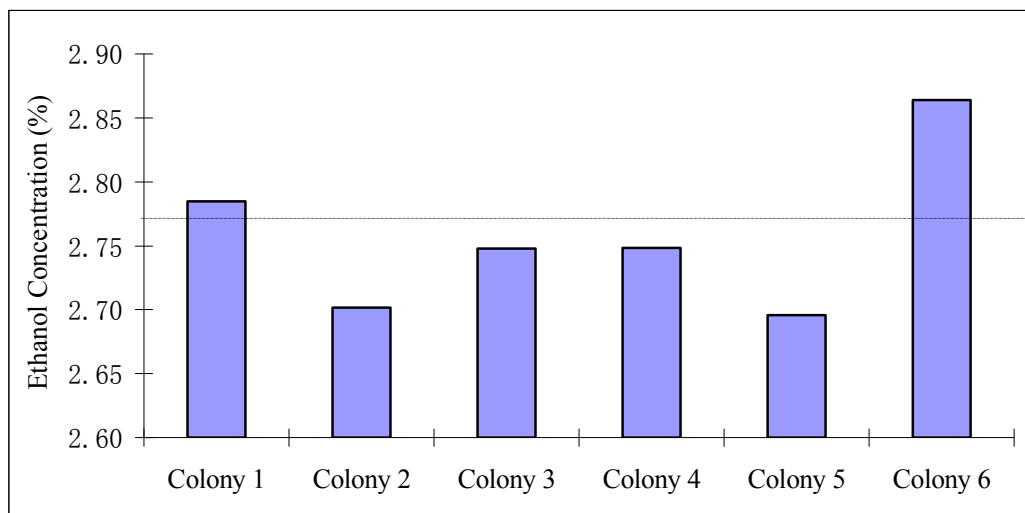


Fig. 5.7 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of six colonies from adaptive mutation with 1.4% acetic acid (Horizontal lines represent the average of six colonies analyzed.)

In summary, an adaptation procedure was developed. Four rounds of adaptation mutation were successfully carried out. Acetic acid tolerant mutants were successfully developed using the adaptation method. The best mutant tolerated 14 g/l acetic acid.

It was observed that as acetic acid concentration increases, the specific growth rate and final O.D. decrease, reflecting the inhibitory effects of the acetic acid on cell growth, and final cell yield.

The ethanol yield, however, are rather constant, close to theoretical yield. Variations were observed, sometime over 100% theoretical yield, most likely due to the variations of initial glucose concentrations.

5.1.2 Adaptive mutation by reducing pH

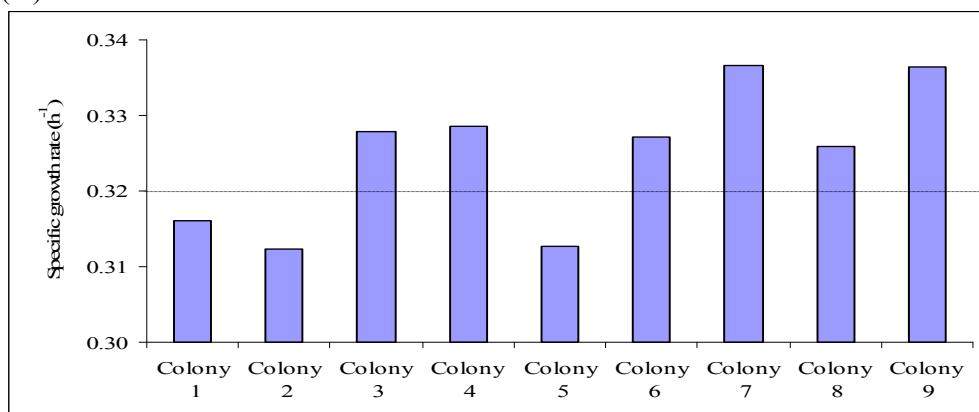
As discussed in chapter 3, the toxicity of acetic acid is much more severe at low pH due to a higher percentage of undissociative species at lower pH. For example, the ratio

of $[\text{HAc}]_{\text{pH } 6}/[\text{HAc}]_{\text{pH } 5}$ is around 1/6 at any acetic acid concentration. The previous rounds of adaptation were carried out at pH 6.0. In order to generate better mutants, adaptation was also performed at lower pH. Mutant ZM6010 was chosen as the starting point because it was adapted in 1.0% acetic acid, which was an intermediate acetic acid concentration. If the concentration is too low, the effect of pH on the growth inhibition would be small, but if the concentration is too high, the effect would be too severe for cells to overcome. When comparing the specific growth rate, this mutant was also at a changing point. The specific growth rate was around 0.4 h^{-1} for both mutant ZM 6002 and ZM6005, but the specific growth rate was only around 0.33 h^{-1} for mutant ZM6010. This means in the presence of 1.0% acetic acid, the acetic acid started to affect the growth of cells. So ZM6010 would be a good choice as the starting point for adaptation by reducing pH.

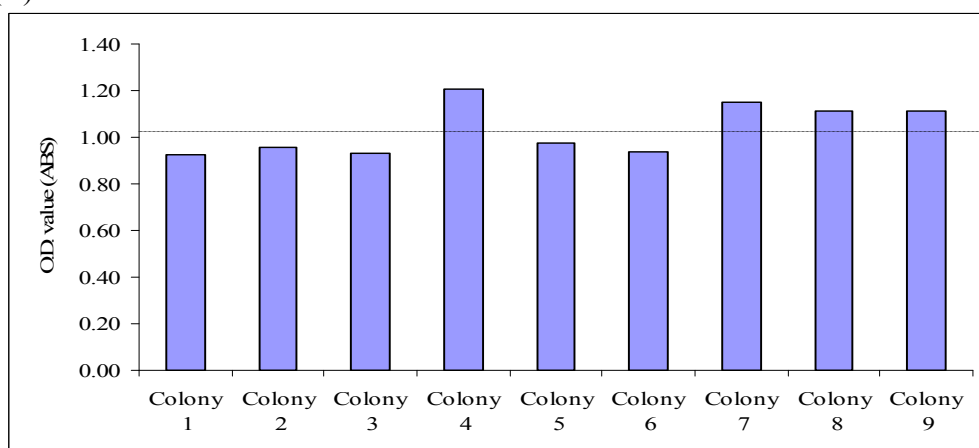
This adaptation was carried out in essentially same way as previous rounds except the pH was lowered to 5.5. After adaptation, single colonies were obtained, and were subjected to the same screening procedure as previous rounds.

Figure 5.8 shows the screening results of the nine colonies selected from plate containing 1.0% acetic acid at pH 5.5. Based upon data presented in Figure 5.8, colony 7 was selected for further adaptive mutation. This mutant was named ZM5510. The glycerol stocks were made for this strain and stored in the -80°C freezer.

(A)



(B)



(C)

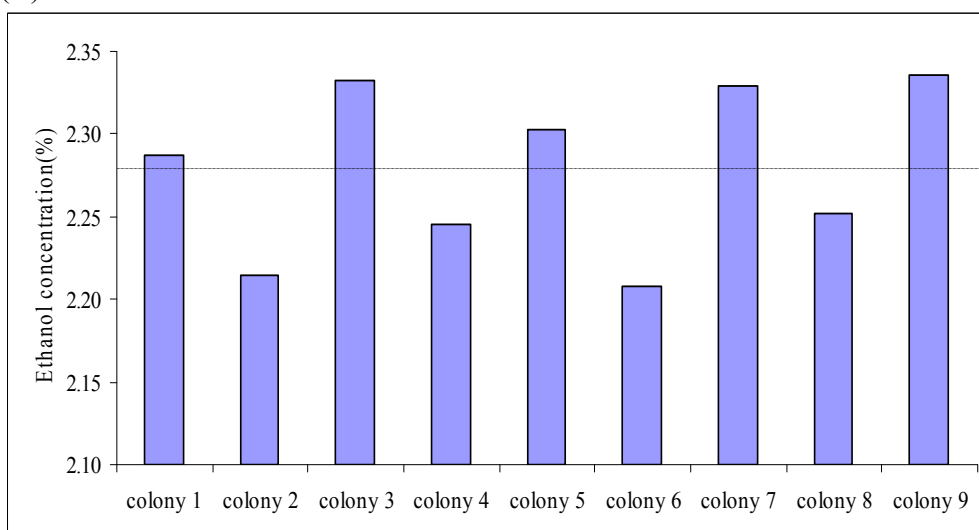
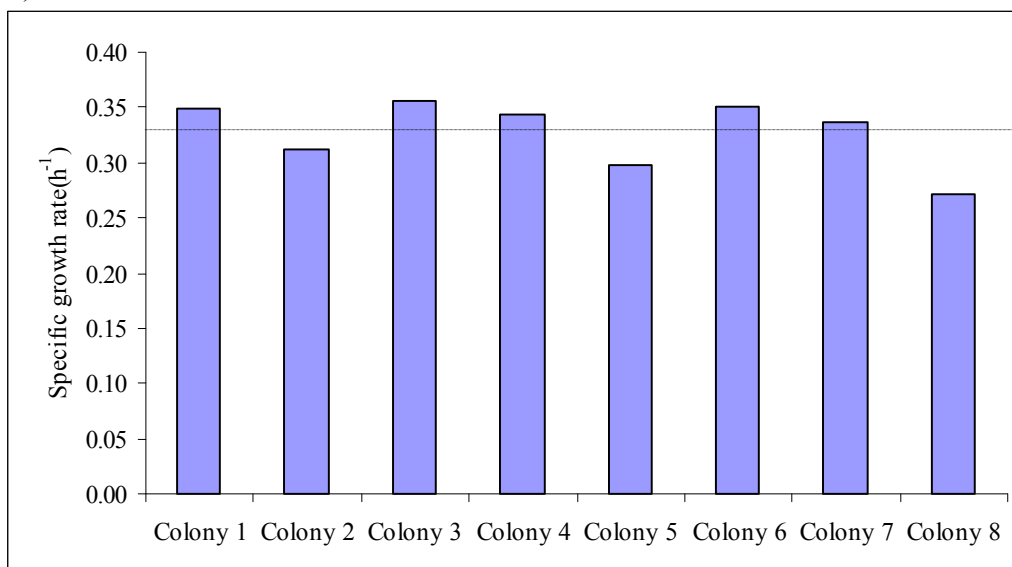


Fig. 5.8 Specific growth rate and ethanol concentration for nine colonies from adaptive mutation at pH 5.5 in the presence of 1.0% acetic acid (Horizontal lines represent the average of nine colonies analyzed.)

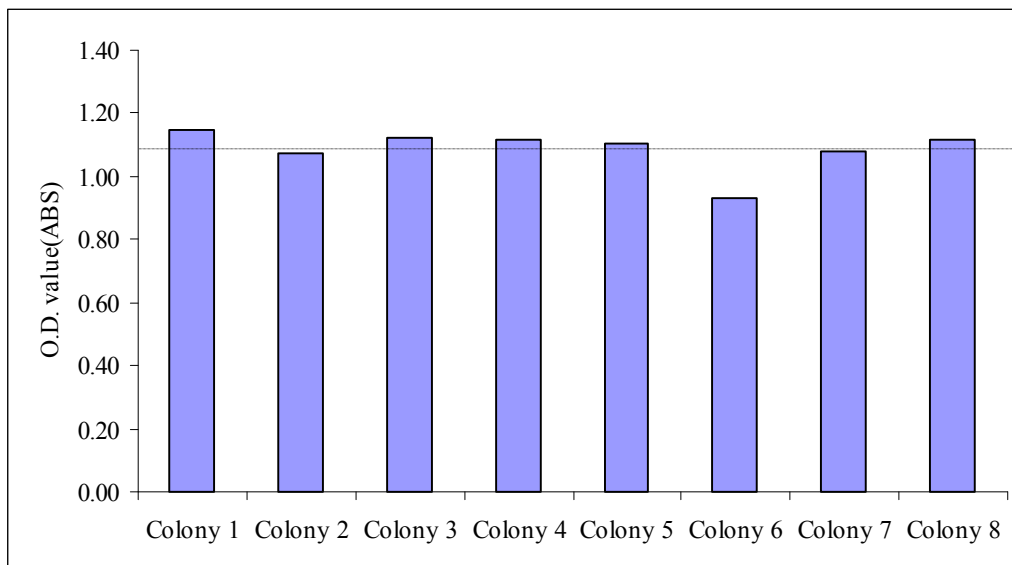
Mutant ZM5510 was further adapted in the medium with 1.0% acetic acid at pH 5.0. Figure 5.9 shows the specific growth rate, O.D. value and ethanol concentration after 24 hours fermentation for eight single colonies picked from agar plate at pH 5.0 in the presence of 1.0% acetic acid. Colony 3 was chosen for further investigation, and was named ZM5010. The glycerol stocks were made for this strain and stored in the -80°C freezer.

Attempts were also made to adapt ZM5510 to higher concentrations of acetic acid (1.2%, 1.4%), and at two pHs (pH 5.0 and pH 5.5). However no single colony grew on the agar plate under these conditions. It appears that, at pH 5.0 and pH 5.5, concentration of 1.0% acetic acid was the limit.

(A)



(B)



(C)

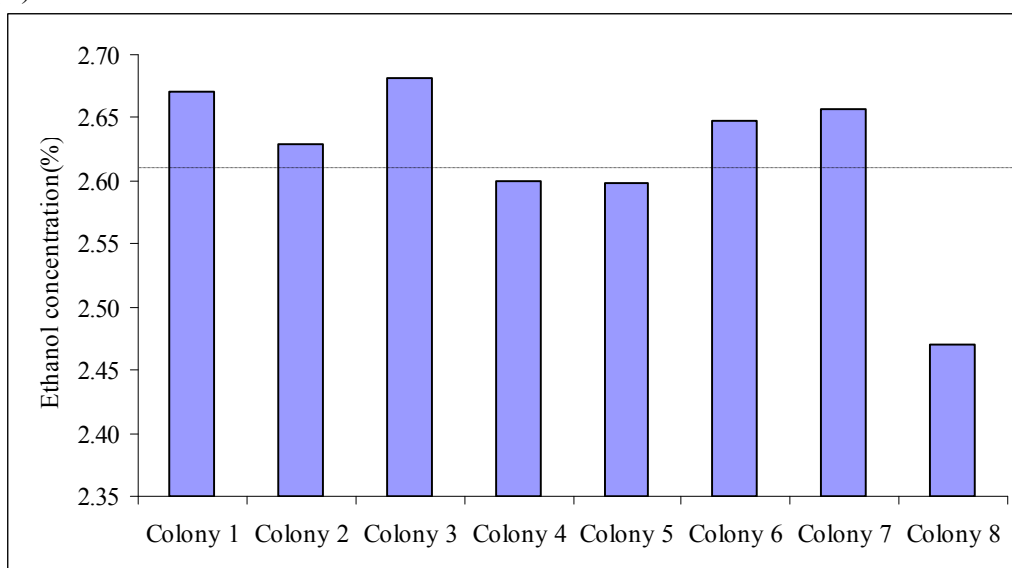


Fig. 5.9 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of eight single colonies from adaptive mutation at pH 5.0 in the presence of 1.0% acetic acid (Horizontal lines represent the average of eight colonies analyzed.)

Table 5.1 summarizes mutants obtained so far. “+” means the mutant in this condition was successfully developed. “-” means no mutant was developed in this condition. “x” means no experiment was carried out under this condition.

Table 5.1 Summary of the mutants developed by adaptive mutation

Acetic acid concentration	0.2%	0.5%	1.0%	1.2%	1.4%	1.6%
pH=6.0	+	+	+	+	+	-
pH=5.5	x	x	+	-	-	-
pH=5.0	X	x	+	-	-	-

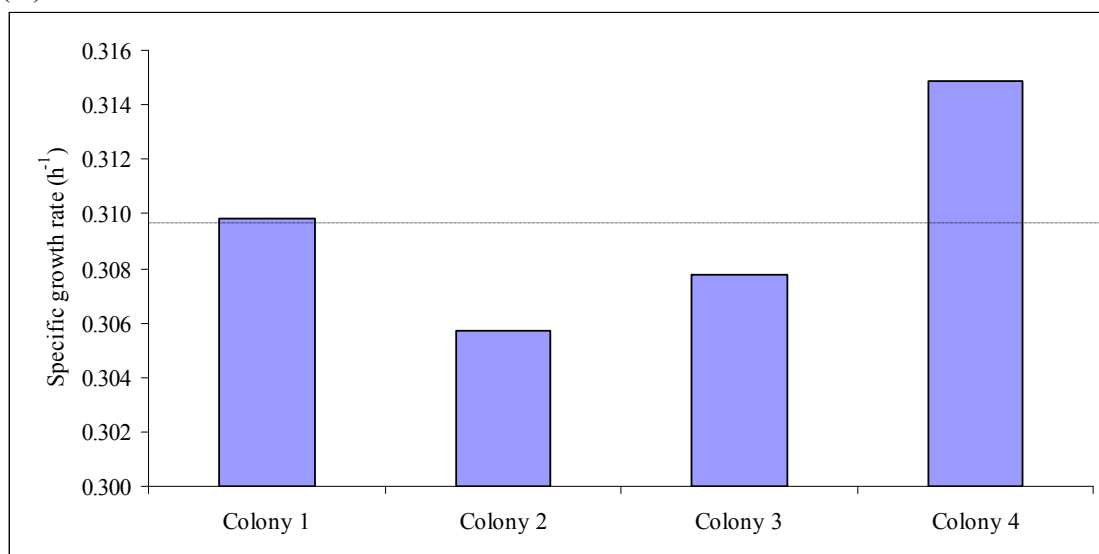
5.2 NTG mutagenesis

As stated in the literature review, NTG mutagenesis was used previously by other researchers to develop acetic acid tolerant strain. To improve the acetic acid tolerance of our mutants, the selected mutant was further treated by NTG mutagenesis. The seed strain was mutant ZM5510, because this strain was developed at an intermediate pH and intermediate acetic acid concentration. Following NTG mutagenesis as described in chapter 4, the cultures were plated on agar plates containing different concentrations of acetic acid (1.0%, 1.2%, 1.4% and 1.6%) and at different pHs (5.0, 5.5 and 6.0). Single colonies were formed on the agar plates at each acetic acid concentration at pH 5.5 and 6.0. However, single colony was only formed on the solid medium with 1.0% acetic acid concentration at pH 5.0. The colonies were small and it was difficult to tell which one was bigger with naked eyes. There were only a few colonies on each plate, hence, only three or four single colonies were picked for screening.

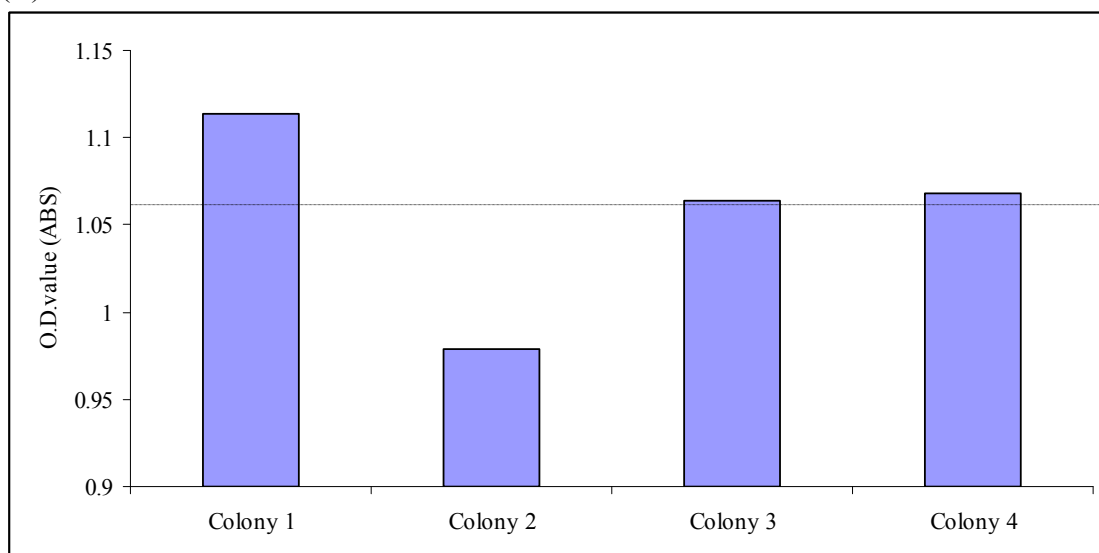
Figure 5.10 shows the specific growth rate, O.D. value and ethanol concentration of the four single colonies. The differences between these four candidates were significant. This is because that the NTG mutagenesis is a random mutation. The change inside the strain is complex, so one strain can be significantly different from the other. The same

parameters were used to screen the colonies. Colony 4 had a higher specific growth rate and ethanol concentration than average value. And its O.D. value was around the average value, so it was chosen for further investigation. The glycerol stocks were made for this strain and stored in the -80°C freezer. This mutant was named as ZMNTG6014.

(A)



(B)



(C)

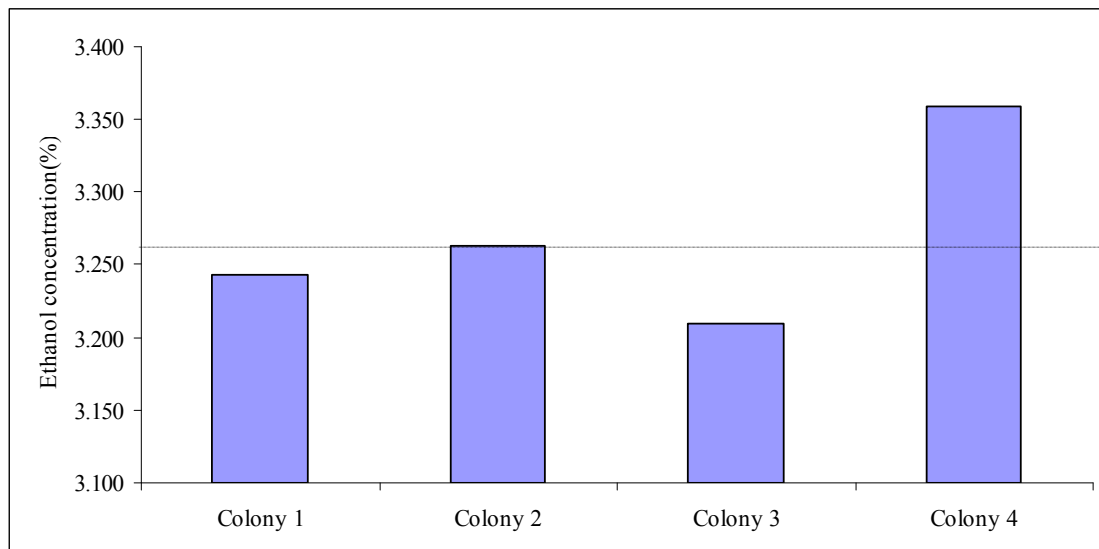
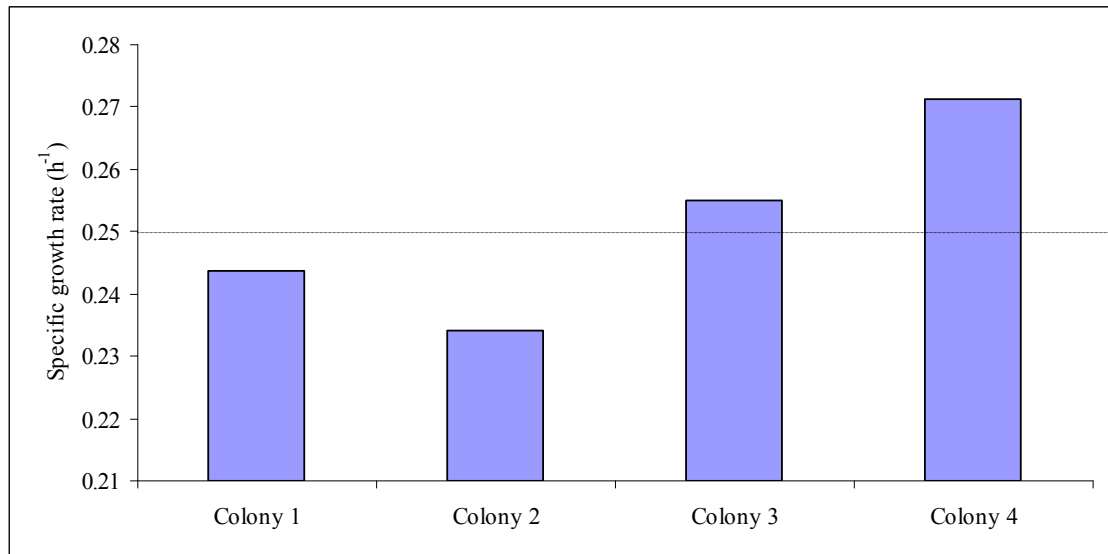


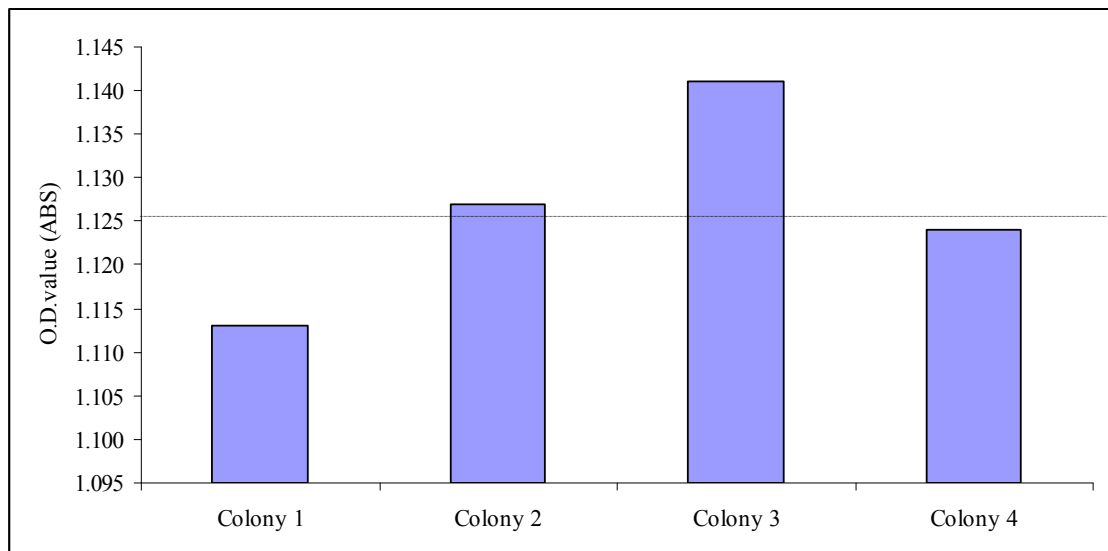
Fig. 5.10 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of four single colonies from NTG mutagenesis at pH 6.0 and in the presence of 1.4% acetic acid (Horizontal lines represent the average of four colonies analyzed.)

Figure 5.11 shows the characteristics of the four single colonies from NTG mutagenesis at pH 6.0 in the presence of 1.6% acetic acid. The performance of these four single colonies was different. Colony 4 had higher specific growth rate than average value. But the other two parameters (O.D. and ethanol concentration) of this colony were around the average value. Colony 3 had the highest O.D. value, which was above the average value. And the ethanol concentration of this colony was also above the average. But its specific growth rate was around the average area. Colony 2 had highest ethanol concentration, which was higher than average, but its specific growth rate and O.D. value were all around the average value. Because the specific growth rate was always thought as the most important parameter, colony 4 was chosen for further investigation. The glycerol stocks were made for this strain and stored in the -80°C freezer. This mutant was named as ZMNTG6016.

(A)



(B)



(C)

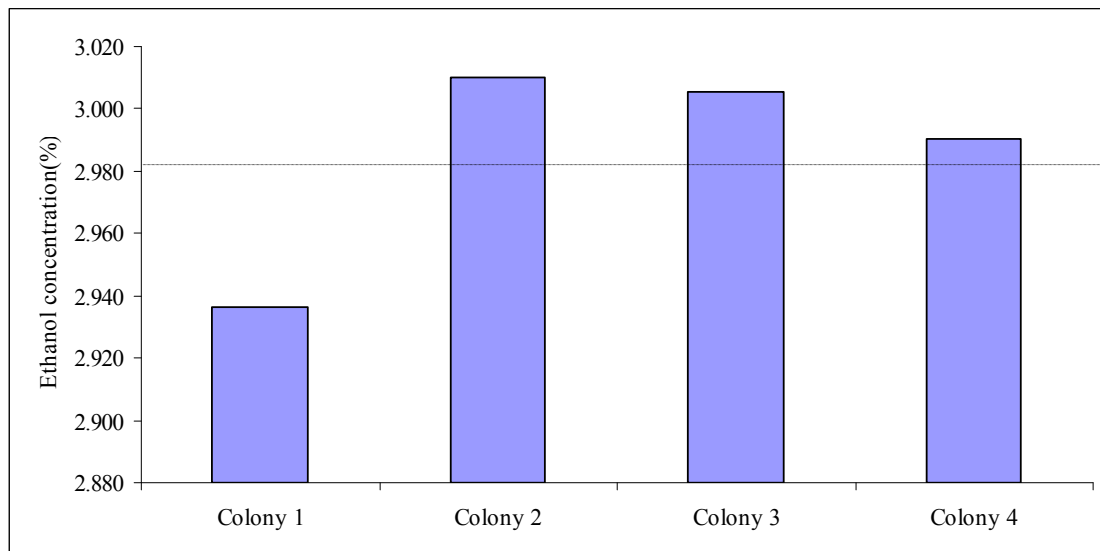
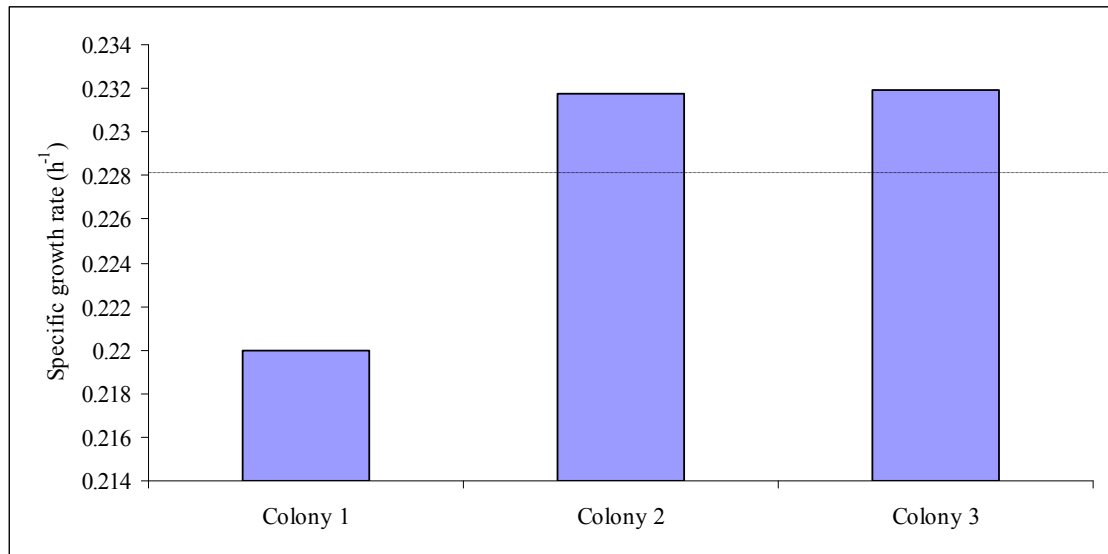


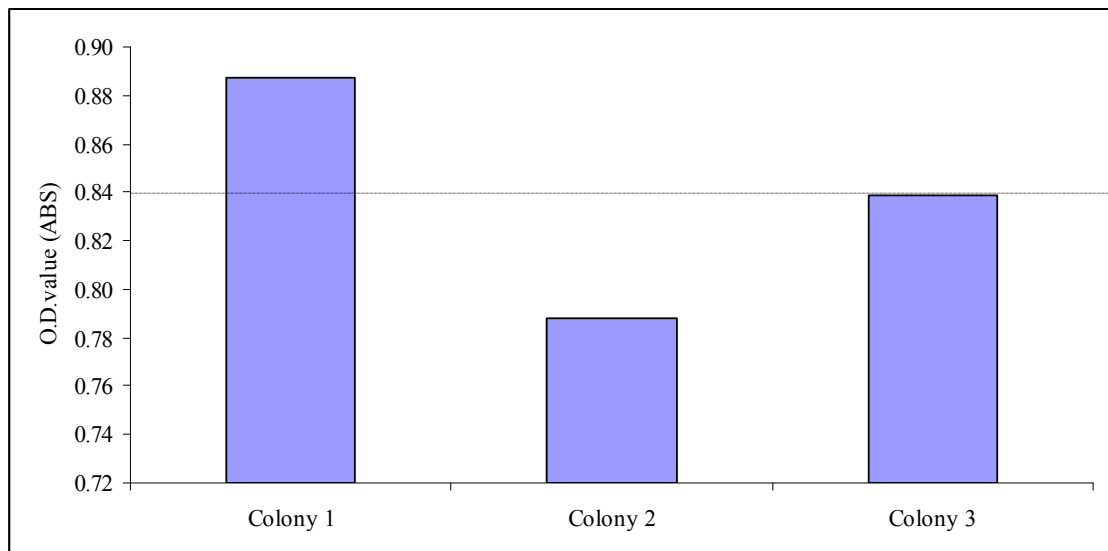
Fig. 5.11 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of four single colonies from NTG mutagenesis at pH 6.0 and in the presence of 1.6% acetic acid (Horizontal lines represent the average of four colonies analyzed.)

Figure 5.12 shows the specific growth rate and O.D. value of three colonies from NTG mutagenesis at pH 5.5 in the presence of 1.4% acetic acid. The specific growth rate of colony 1 was lower than average value, so this strain was not considered further. Colony 2 and colony 3 had almost the same specific growth rate. And the O.D. values of these two colonies were all around the average values. Because the colony 3 had a little higher O.D. value than colony 2, colony 3 was chosen for further investigation and named as ZMNTG5514. However the glycerol stock were prepared for both strains and stored in the -80°C freezer.

(A)



(B)



(C)

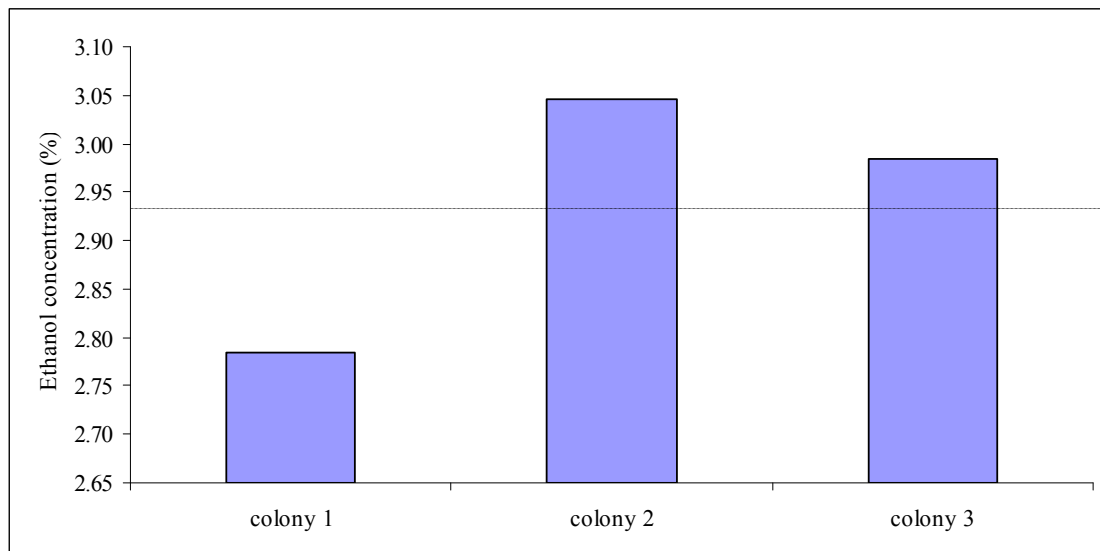
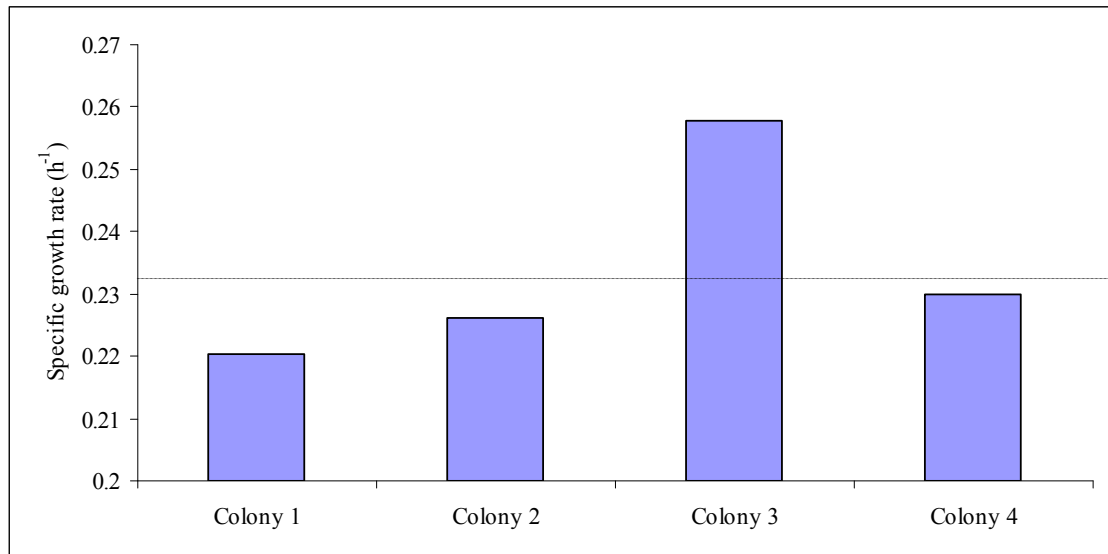


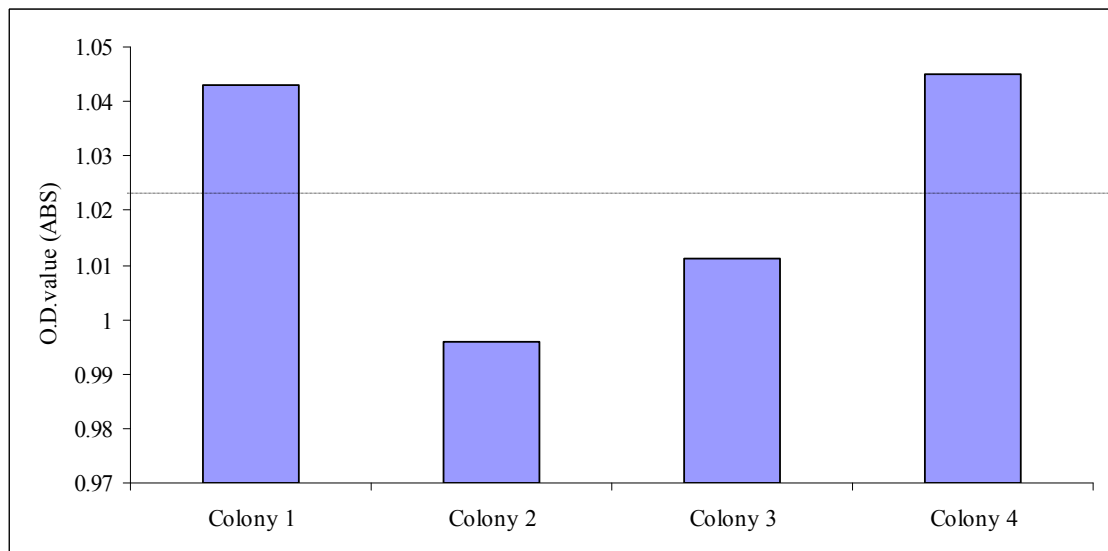
Fig. 5.12 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of four single colonies from NTG mutagenesis at pH 5.5 and in the presence of 1.4% acetic acid (Horizontal lines represent the average of three colonies analyzed.)

Figure 5.13 shows the parameters of the four single colonies from NTG mutagenesis at pH 5.5 in the presence of 1.6% acetic acid. Because of the low pH and high acetic acid concentration, these four single colonies exhibited significant differences. Colony 3 was chosen for further investigation. The glycerol stocks were made for this strain and stored in the -80°C freezer. This mutant was named as ZMNTG5516.

(A)



(B)



(C)

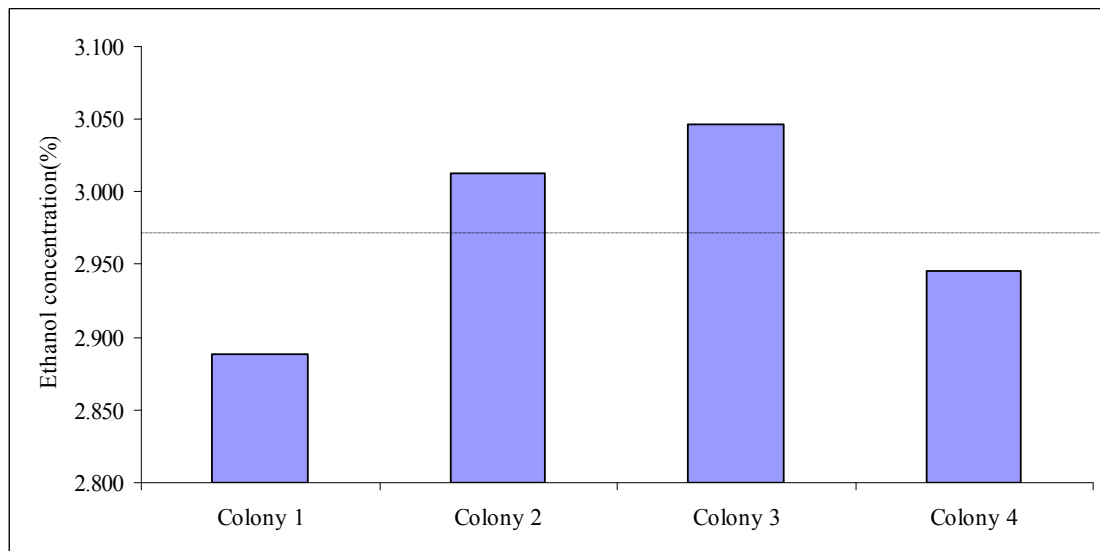


Fig. 5.13 (A) Specific growth rate (B) O.D. value and (C) Ethanol concentration of four single colonies from NTG mutagenesis at pH 5.5 and in the presence of 1.6% acetic acid (Horizontal lines represent the average of four colonies analyzed.)

CHAPTER 6 MUTANT CHARACTERIZATION

In this chapter, the characteristics of wild type strain and the selected mutants are compared. Specific growth rate, O.D. value and ethanol concentration are chosen as three parameters to represent the characteristics of the strains because of their importance for cell growth and ethanol production. The effects of other inhibitors are also studied.

6.1 The characteristics of wild type strain and mutants obtained by adaptive mutation

The acetic acid could affect the growth of a microorganism in three aspects: decreasing the specific growth rate, decreasing the final cell concentration (represented by O.D.), and increasing the lag phase. The characteristics of mutant ZM6010, ZM6014, ZM5510 and ZM5010 were compared to those of wild type strain. ZM6010 and ZM6014 were the mutants adapted at high acetic acid concentration; ZM5510 and ZM5010 were the mutants adapted at the low pH. The growth curves of these five strains at different acetic acid concentrations and pHs are shown in Figure 6.1 to 6.12. The lag phase and specific growth rate of these strains under different conditions are summarized in Table 6.1 and 6.2.

Figure 6.1 shows the O.D. values of these strains as a function of time at pH 6.0 without acetic acid. Under this condition, the performances of all five strains are quite similar. The lag phase is about 4.1 hours; the specific growth rate is about 0.52 h^{-1} ; and the final O.D. value is about 1.5. All the strains completed the fermentation within one day.

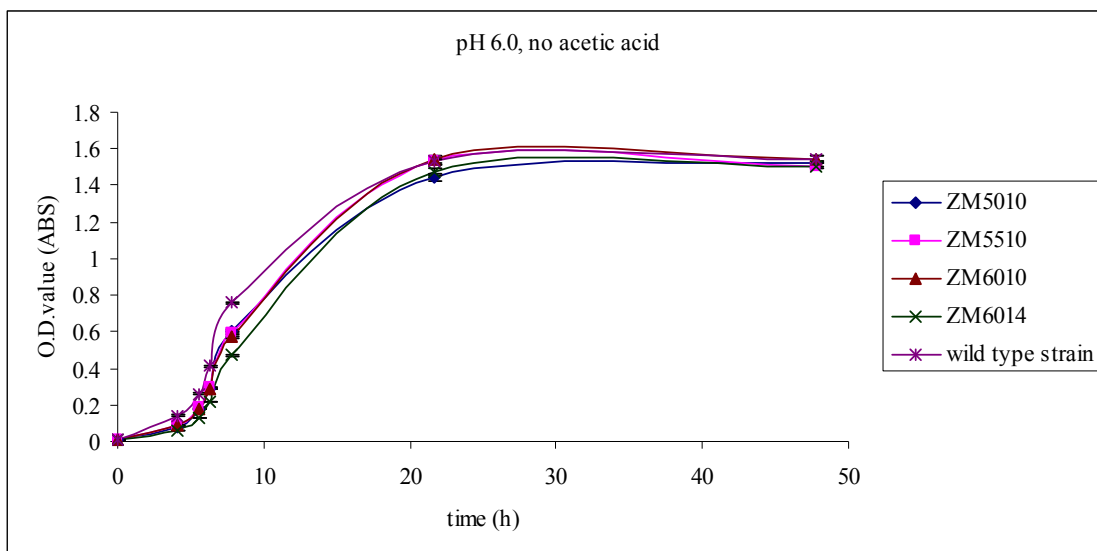


Fig. 6.1 Growth curves at pH 6.0 in the absence of acetic acid

In the presence of acetic acid, there are significant differences between the mutants and the wild type strain. In the presence of 1.0% acetic acid (Figure 6.2), all the strains have longer lag phase and lower specific growth rate compared to the condition in the absence of acetic acid. The wild type strain has a longer phase, and lower specific growth rate compared to the mutants. The differences between the mutants are small. Under this condition, the lag phase is about 5.0 hours for all mutants, but 7.8 hours for wild type strain. The specific growth rate of all mutants is about 0.43 h^{-1} , but the specific growth rate of wild type strain is only 0.31 h^{-1} . The O.D. of all mutants is still above 1.1, but this value of wild type strain is lower than 1.0. Both the mutants and the wild type strain could still complete the fermentation in one day.

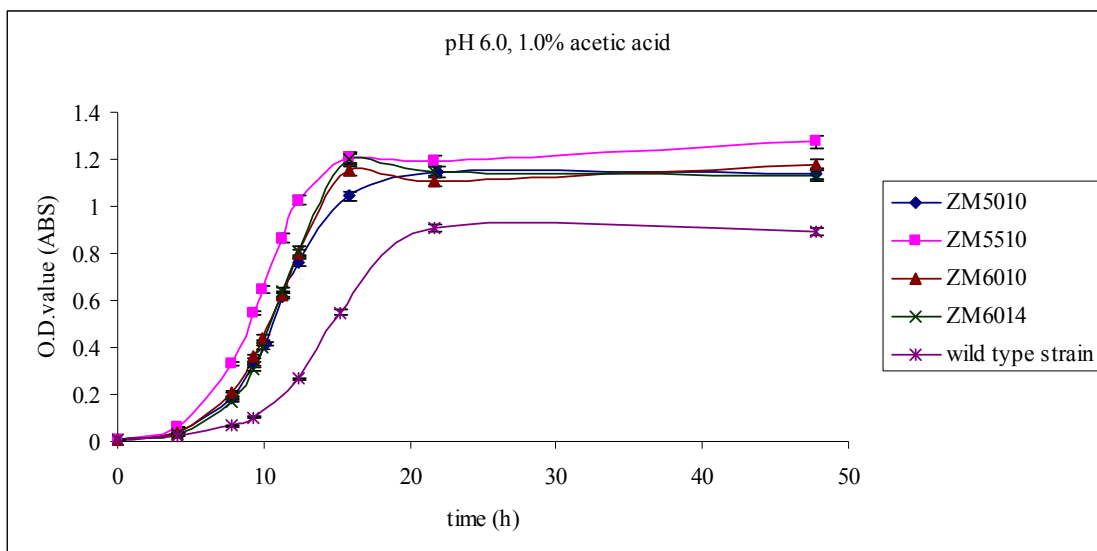


Fig. 6.2 Growth curves at pH 6.0 in the presence of 1.0% acetic acid

With increasing acetic acid concentration to 1.2%, the specific growth rate decreases and lag phase increases further for all strains. The difference between the mutants and the wild type strain becomes larger. Under this condition, the wild type strain could not complete the fermentation in two days. And the lag phase of wild type strain increases to 15.2 hours. The specific growth rate of the wild type strain decreases to 0.151 h^{-1} . On the other hand, all the mutants completed the fermentation in one day. And lag phase is about 5.5 hours for all mutants. The specific growth rates and O.D. of the mutants differ significantly. ZM6014 and ZM5510 have a slightly higher specific growth rate, which is about 0.43 h^{-1} , than that of ZM5010 and ZM6010, which is about 0.36 h^{-1} . The O.D.s of ZM6014 and ZM5510 are also a slightly higher than ZM5010 and ZM6010.

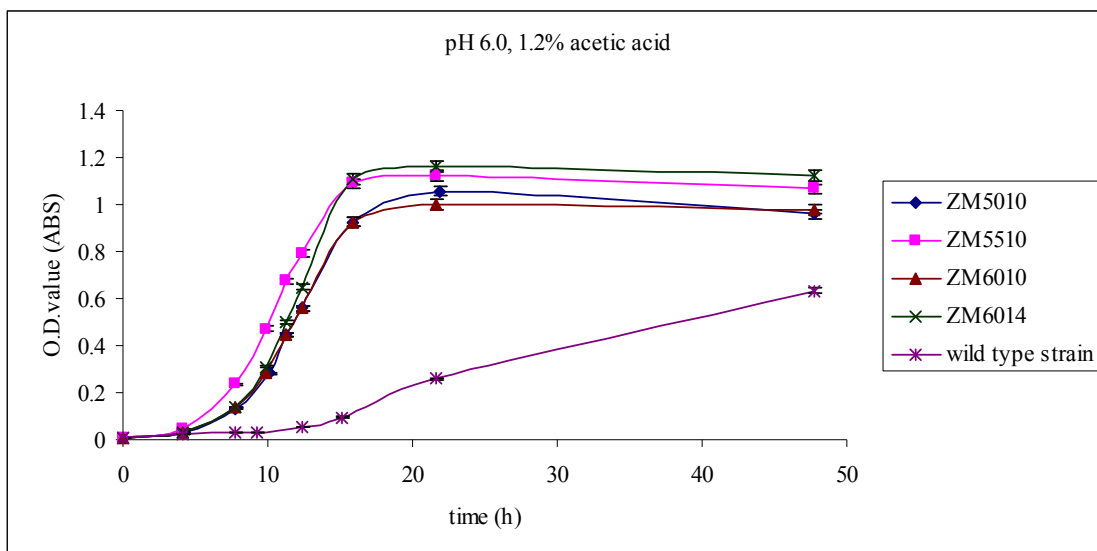


Fig. 6.3 Growth curves at pH 6.0 in the presence of 1.2% acetic acid

In the presence of 1.4% acetic acid (Figure 6.4), the effect of acetic acid on cell growth becomes much severer. The wild type strain barely grows in two days. The O.D. of wild type strain at 48 hours is only 0.142 and the lag phase of wild type train is as long as 40 hours. While the lag phase of all mutants is quite similar (about 7.8 hours), the differences between mutants become much larger. ZM6014 has the highest specific growth rate, followed by ZM5510, ZM5010. ZM5510 has the highest O.D, followed by ZM6014 and ZM5010. ZM6010 has both the lowest specific growth rate and lowest O.D. value. This makes sense. ZM6014 was adapted at higher acetic acid concentration than ZM6010 and ZM5510, ZM5010 were adapted at lower pH than ZM6010. So it is not surprising to see ZM6010 does not perform as well as other mutants at high acetic acid concentration.

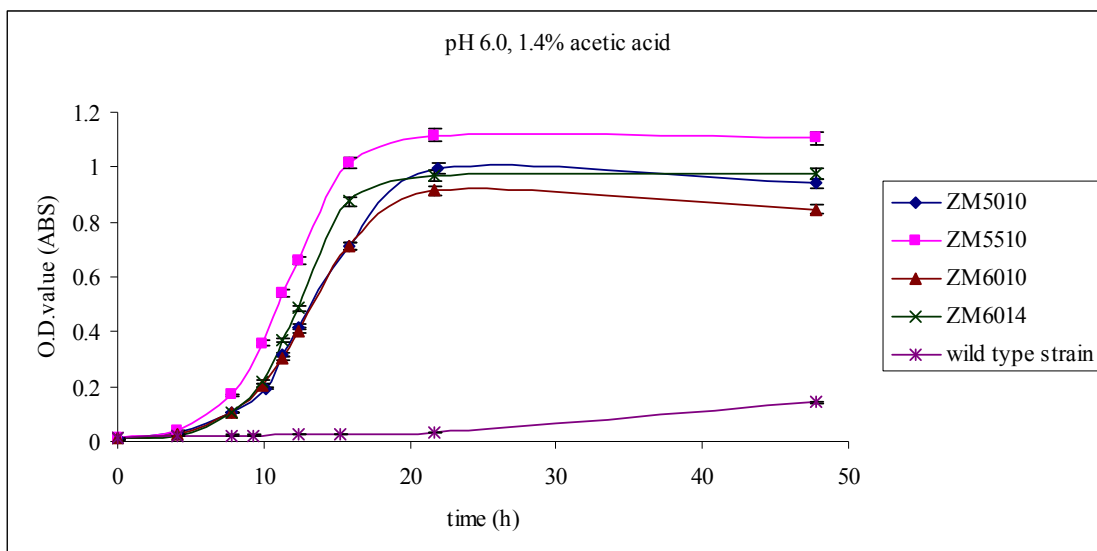


Fig. 6.4 Growth curves at pH 6.0 in the presence of 1.4% acetic acid

The experimental results presented above clearly show that the mutants reached by adaptive mutation have higher specific growth rate, shorter lag phase and higher O.D.s in the presence of high concentration of acetic acid, indicating higher acetic acid tolerance for mutants than the wild type strain. We can conclude that the adaptive mutation method could successfully develop high acetic acid tolerant *Z. mobilis* strains.

Figures 6.5 to 6.8 show the growth curves of wild type strain and mutants at pH 5.5 with different acetic acid concentrations.

In the absence of acetic acid, the performances of wild type stain and mutants are similar. Their performances are also similar to that at pH 6.0. Under this condition, the specific growth rate of all strains is about 0.52 h^{-1} ; the O.D. is about 1.5; and the lag phase is about 4.1 hours.

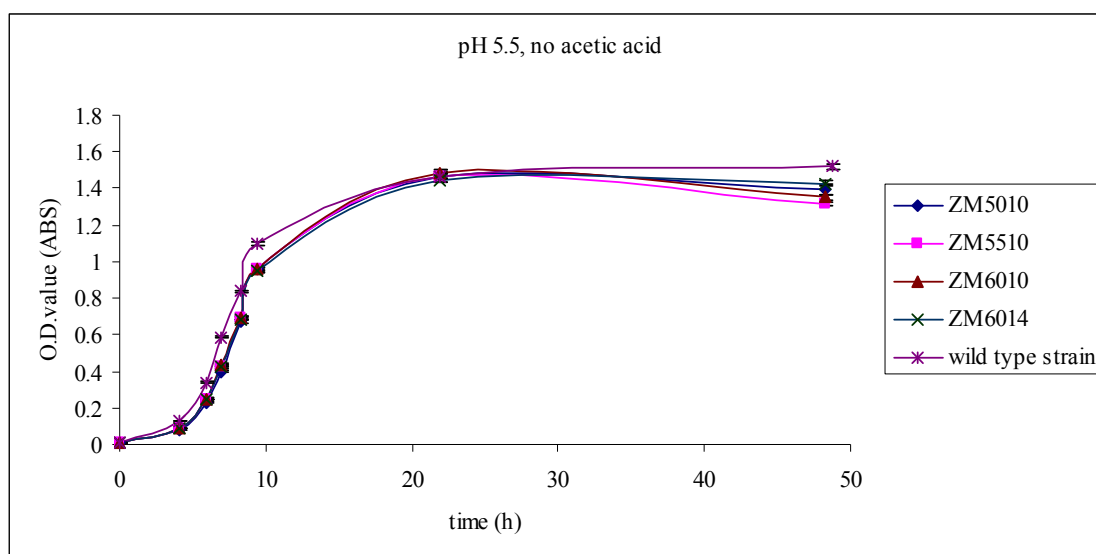


Fig. 6.5 Growth curves at pH 5.5 in the absence of acetic acid

With increasing the acetic acid concentration, the final O.D. and the specific growth rate decrease and the lag phase increases. Compared to the results at pH 6.0, the specific growth rates and final O.D.s decrease and the lag phase increases in the same amount of acetic acid. For wild type strain, the lag phase is 8.25 hours in the presence of 1.0% acetic acid. The specific growth rate is 0.306 h^{-1} under this condition and the final O.D. is about 0.9. The four mutants have a similar lag phase (5.0 hours), and similar final O.D.s. But ZM6010 has the lowest specific growth rate 0.354 h^{-1} , while the specific growth rates of other three mutants are all around 0.42 h^{-1} .

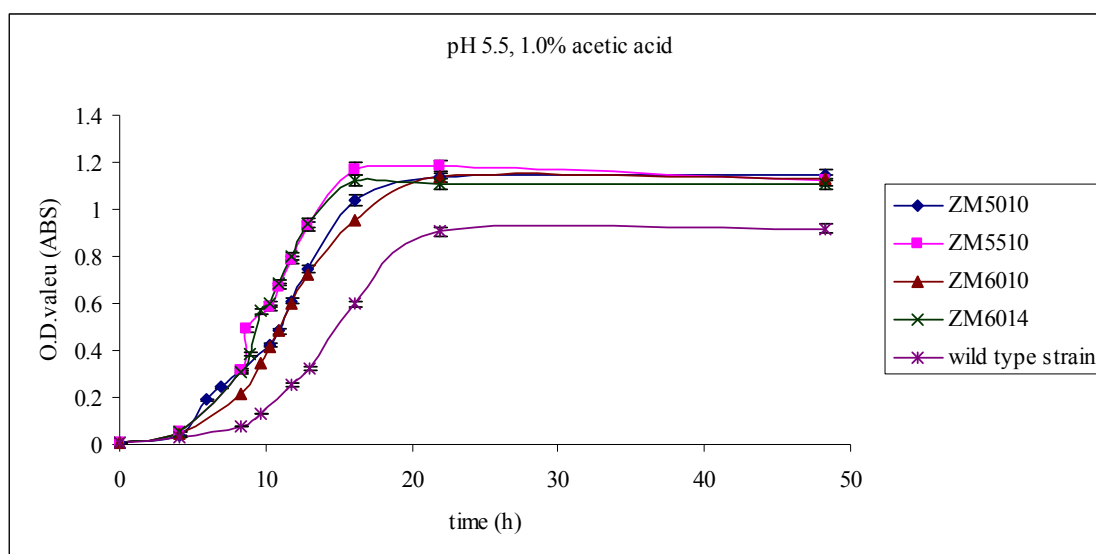


Fig. 6.6 Growth curves at pH 5.5 in the presence of 1.0% acetic acid

In the presence of 1.2% acetic acid, the lag phase of the wild strain increases to 16 hours. The specific growth rate decreases to 0.103 h^{-1} . And it could not complete the fermentation in two days. Among the four mutants, ZM6010 has longest lag phase and lowest specific growth rate. ZM5010 has lower specific growth rate than ZM6014 and ZM5510, although the lag phase is similar for these three mutants.

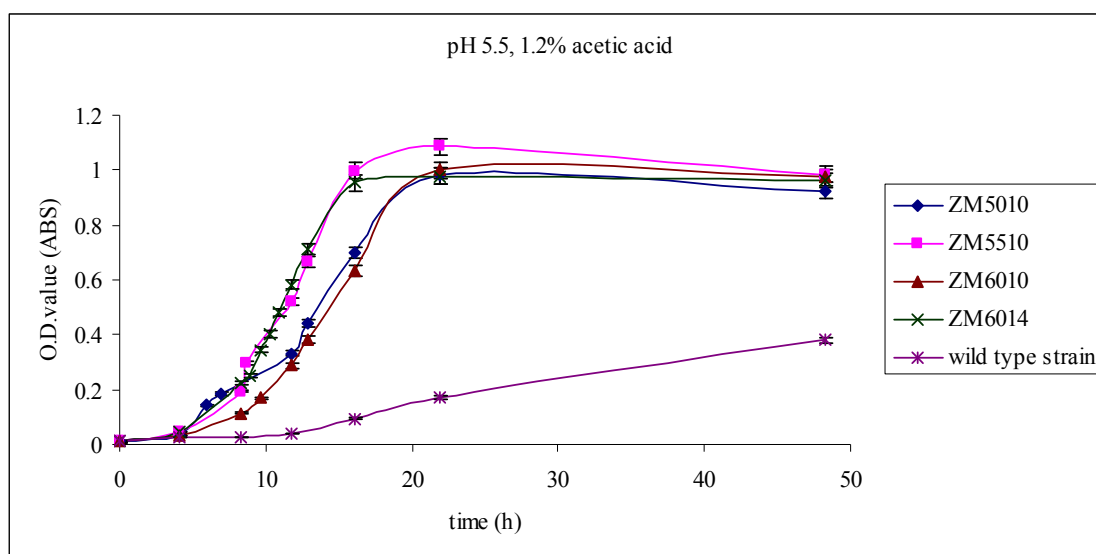


Fig. 6.7 Growth curves at pH 5.5 in the presence of 1.2% acetic acid

In the presence of 1.4% acetic acid, wild type strain did not grow at all in two days. And the differences between the mutants became much larger. ZM6014 has the highest O.D. and specific growth rate, both of which are slightly higher than ZM5510. The O.D. and specific growth rate of ZM5010 are lower than both ZM6014 and ZM5510. ZM6010 has the lowest O.D. and specific growth rate, consistent with the results shown above.

The experimental results at pH 5.5 further prove that the mutants have higher acetic acid tolerance than wild type strain. The results obtained from the experiments carried out at pH 5.5 in the presence of 1.4% acetic acid confirm that ZM6010 has lowest acetic acid tolerance among four mutants. And ZM5010 has lower acetic acid tolerance than ZM6014 and ZM5510. This conclusion is not as expected, because in principle, the mutant ZM5010, which was adapted at lower pH than ZM5510, should have the higher acetic acid tolerance than ZM5510. This unexpected result may be explained as follows. At pH5.0, the sizes of single colonies growing on the agar plate were small and were similar to each other. So it was very difficult to tell which was bigger than others with

naked eyes. The best single colonies were missed at that time. Another possible reason is that the mutant ZM5010 is weaker than ZM5510. Although it could tolerate the acetic acid, it could not maintain the same specific growth rate as ZM5510.

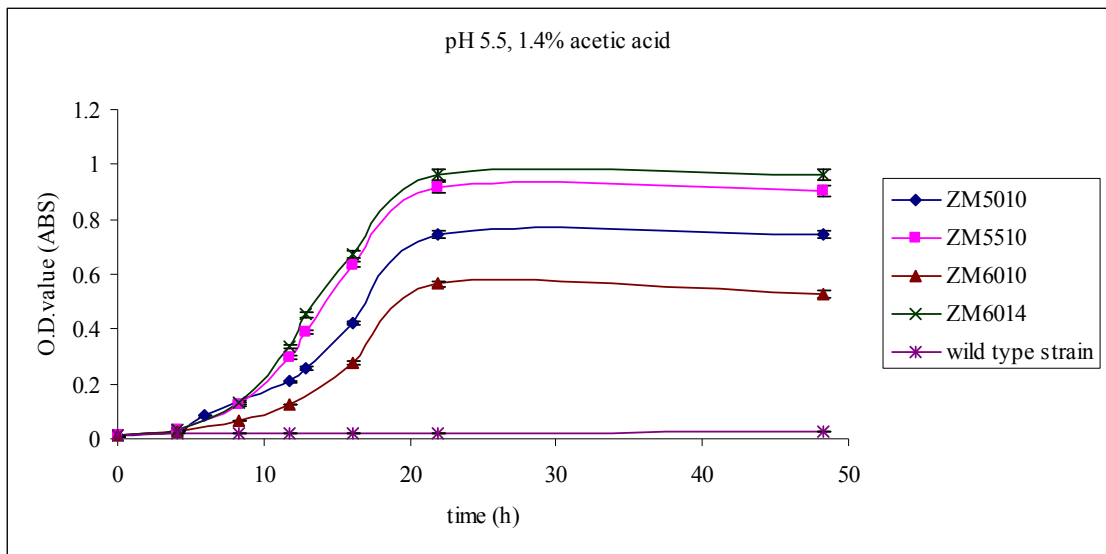


Fig. 6.8 Growth curves at pH 5.5 in the presence of 1.4% acetic acid

Strains were also evaluated at pH 5.0 (Figures 6.9-6.12). At pH 5.0, the performances of mutants and wild strain are similar to those at pH 5.5 and pH 6.0, in the absence of acetic acid. The specific growth rate is about 0.52 h⁻¹; the lag phase is about 0.42 hours; the O.D. is about 1.5.

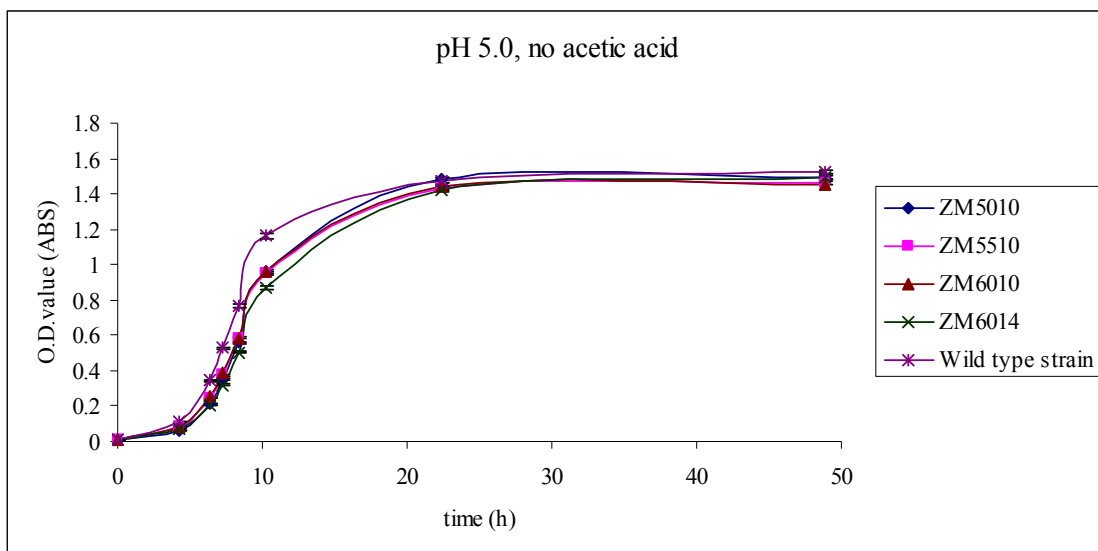


Fig. 6.9 Growth curves at pH 5.0 in the absence of acetic acid

In the presence of 1.0% acetic acid, wild type strain has the longest lag phase, lowest specific growth rate and lowest O.D. value. Under this condition, although the lag phases for the four mutants are similar, the specific growth rate of ZM6010 is much lower than that of other mutants, 0.21 h^{-1} , versus 0.32 h^{-1} .

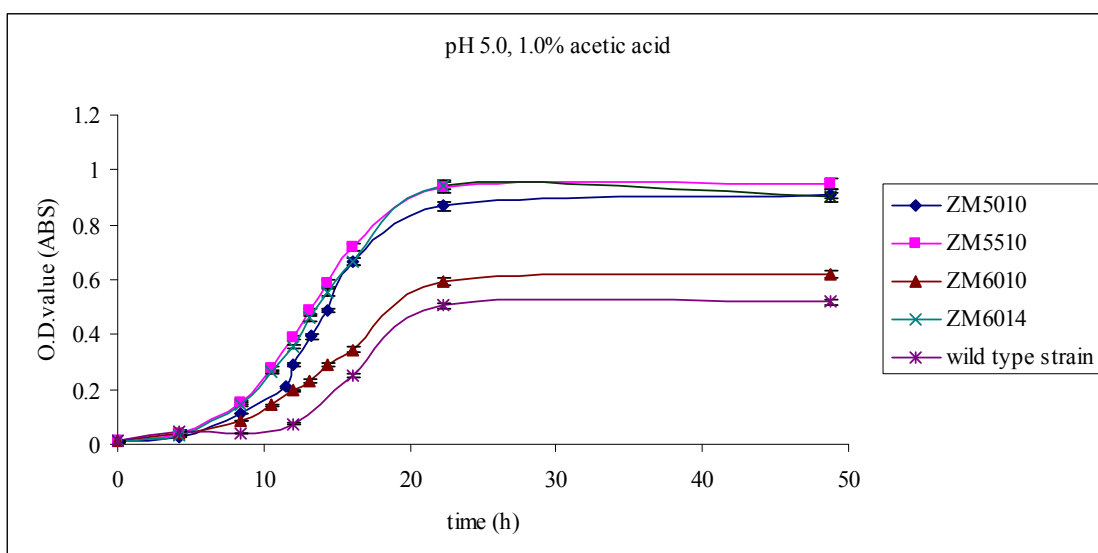


Fig. 6.10 Growth curves at pH 5.0 in the presence of 1.0% acetic acid

In the presence of 1.2% acetic acid, neither ZM6010 nor wild strain could complete the fermentation in two days. Among ZM5010, ZM5510 and ZM6014, ZM5010 has the lowest specific growth rate, and O.D. value. ZM6014 has the highest specific growth rate, but ZM5510 has the highest O.D. value.

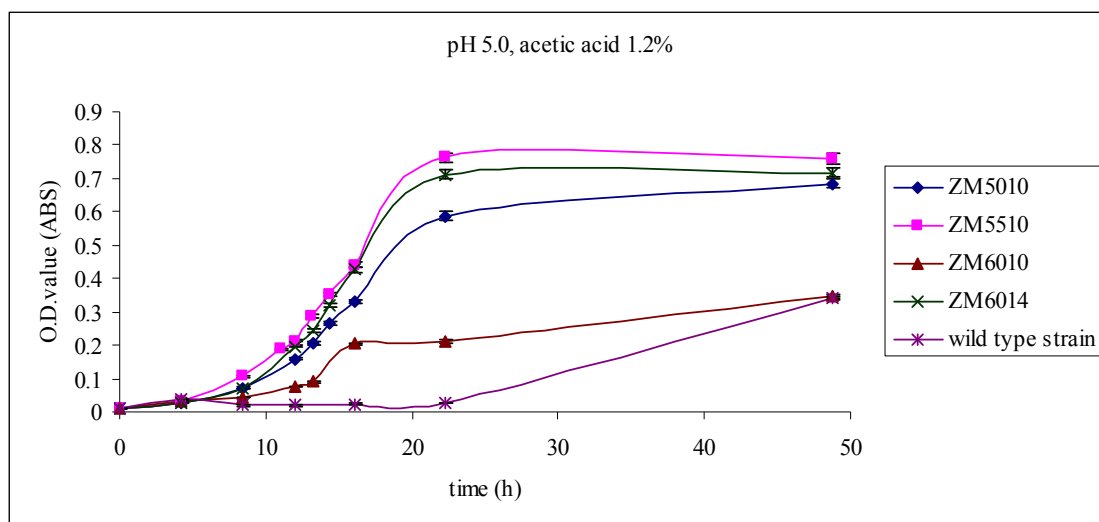


Fig. 6.11 Growth curves at pH 5.0 in the presence of 1.2% acetic acid

In the presence of 1.4% acetic acid, none of the strains completed the fermentation in two days. Wild type strain did not show any growth in two days, and ZM6010 barely grew. Under this severer growth-inhibiting condition, the mutants have a shorter lag phase, a higher specific growth rate and final O.D. than wild type strain. ZM5510, ZM6014 and ZM5010 started to grow around 14 hours, and they exhibited a specific growth rate above 0.1 h^{-1} .

In summary, at this pH, the lag phase also increases with increasing the acetic acid concentration. And both the specific growth rate and O.D. also decreases with increasing the acetic acid concentration. In the presence of same amount of acetic acid, the O.D. and specific growth rate are lower and the lag phase is longer than those at higher pHs for any

strain. These results further support the conclusions stated before. Although it is clear that ZM6010 and ZM5010 are inferiors to the other two mutants, the differences between ZM6014 and ZM5510 are small and insignificant. Both are considered the best mutant from adaptive mutation.

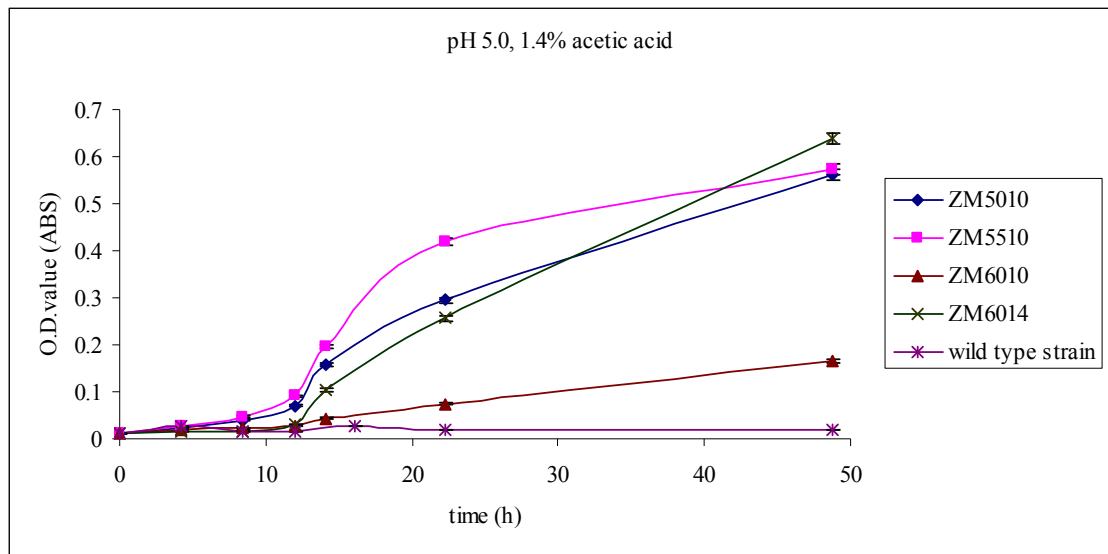


Fig. 6.12 Growth curves at pH 5.0 in the presence of 1.4% acetic acid

Table 6.1 and 6.2 summarizes the lag phase and specific growth of all the strains at different experimental conditions.

Table 6.1 Lag phases (h) of five strains at different pHs and acetic acid concentrations

pH 5.0	Acetic acid concentration			
	0.00%	1.00%	1.20%	1.40%
ZM5010	4.2±0.14	6.3±0.13	8.4±0.17	14.1±0.10
ZM5510	4.2±0.14	6.3±0.13	8.4±0.17	14.1±0.10
ZM6010	4.2±0.14	6.3±0.13	12.5±0.35	22.2±0.23
ZM6014	4.2±0.14	6.3±0.13	8.4±0.17	14.1±0.10
wild type strain	4.2±0.14	12.0±0.36	36.0±0.18	>48
pH 5.5	Acetic acid concentration			
	0.00%	1.00%	1.20%	1.40%
ZM5010	4.1±0.085	5.0±0.10	5.5±0.11	10.6±0.13
ZM5510	4.1±0.085	5.0±0.10	5.5±0.11	10.6±0.13
ZM6010	4.1±0.085	5.0±0.10	8.3±0.16	11.8±0.20
ZM6014	4.1±0.085	5.0±0.10	5.5±0.11	10.6±0.13
wild type strain	4.1±0.085	8.3±0.18	16.0±0.12	>48
pH 6.0	Acetic acid concentration			
	0.00%	1.00%	1.20%	1.40%
ZM5010	4.1±0.070	5.0±0.10	5.5±0.11	7.8±0.15
ZM5510	4.1±0.070	5.0±0.10	5.5±0.11	7.8±0.15
ZM6010	4.1±0.070	5.0±0.10	5.5±0.11	7.8±0.15
ZM6014	4.1±0.070	5.0±0.10	5.5±0.11	7.8±0.15
wild type strain	4.1±0.070	7.8±0.16	15.2±0.15	40.0±0.18

Table 6.2 Specific growth rates (h^{-1}) of five strains at different pHs and acetic acid concentrations

pH 5.0	Acetic acid concentration			
	0.00%	1.00%	1.20%	1.40%
ZM5010	0.535±0.020	0.319±0.008	0.219±0.008	0.119±0.013
ZM5510	0.503±0.005	0.314±0.015	0.252±0.012	0.164±0.012
ZM6010	0.528±0.014	0.211±0.014	0.147±0.008	0.030±0.001
ZM6014	0.507±0.010	0.325±0.010	0.285±0.011	0.135±0.017
wild type strain	0.523±0.021	0.196±0.003	0.098±0.007	N/A
pH 5.5	Acetic acid concentration			
	0.00%	1.00%	1.20%	0.014
ZM5010	0.518±0.015	0.417±0.008	0.304±0.008	0.183±0.007
ZM5510	0.524±0.022	0.415±0.018	0.362±0.017	0.247±0.012
ZM6010	0.525±0.025	0.354±0.020	0.266±0.014	0.169±0.001
ZM6014	0.523±0.022	0.422±0.015	0.386±0.016	0.252±0.014
wild type strain	0.517±0.014	0.306±0.030	0.103±0.002	N/A
pH 6.0	Acetic acid concentration			
	0.00%	1.00%	1.20%	1.40%
ZM5010	0.519±0.017	0.411±0.015	0.363±0.015	0.314±0.006
ZM5510	0.521±0.020	0.447±0.013	0.431±0.010	0.331±0.018
ZM6010	0.516±0.012	0.400±0.030	0.367±0.007	0.289±0.018
ZM6014	0.534±0.019	0.467±0.030	0.425±0.008	0.351±0.007
wild type strain	0.507±0.012	0.306±0.008	0.151±0.017	N/A

In summary, at any pH condition, the lag phase always increases, while the specific growth rate decreases with the increase of acetic acid concentration. At the same acetic acid concentration, the lag phase increases as pH decreases, and the specific growth rate decreases as pH decreases. All the mutants obtained by adaptive mutation have the higher

acetic acid tolerance than wild type strain. Among the mutants, ZM5510 and ZM6014 have the highest acetic acid tolerance, followed by ZM5010 and ZM6010.

6.2 Comparison of mutants developed by adaptive and NTG mutagenesis

The growth behaviors of mutants developed by adaptive mutation alone and by adaptive mutation followed by NTG mutagenesis were compared. Acetic acid concentration 1.4% and 1.6% were chosen as the experimental conditions. pH conditions were 5.0, 5.5 and 6.0. And mutants ZM5510 and ZM6014, the two best mutants from adaptive mutation, were chosen to compare the mutants developed further by NTG mutagenesis.

Figures 6.13 to 6.15 show the growth curves of different mutants in the presence of 1.4% (A) and 1.6% (B) acetic acid at different pHs.

At pH 6.0, in the presence of 1.4% acetic acid, the performances of all the mutants were similar based on the O.D., specific growth rate (0.33 h^{-1}) and lag phase (7.8 hours). However, in the presence of 1.6% acetic acid, ZM5510 has the lower O.D. and specific growth rate (0.249 h^{-1}) than other mutants (0.3 h^{-1}).

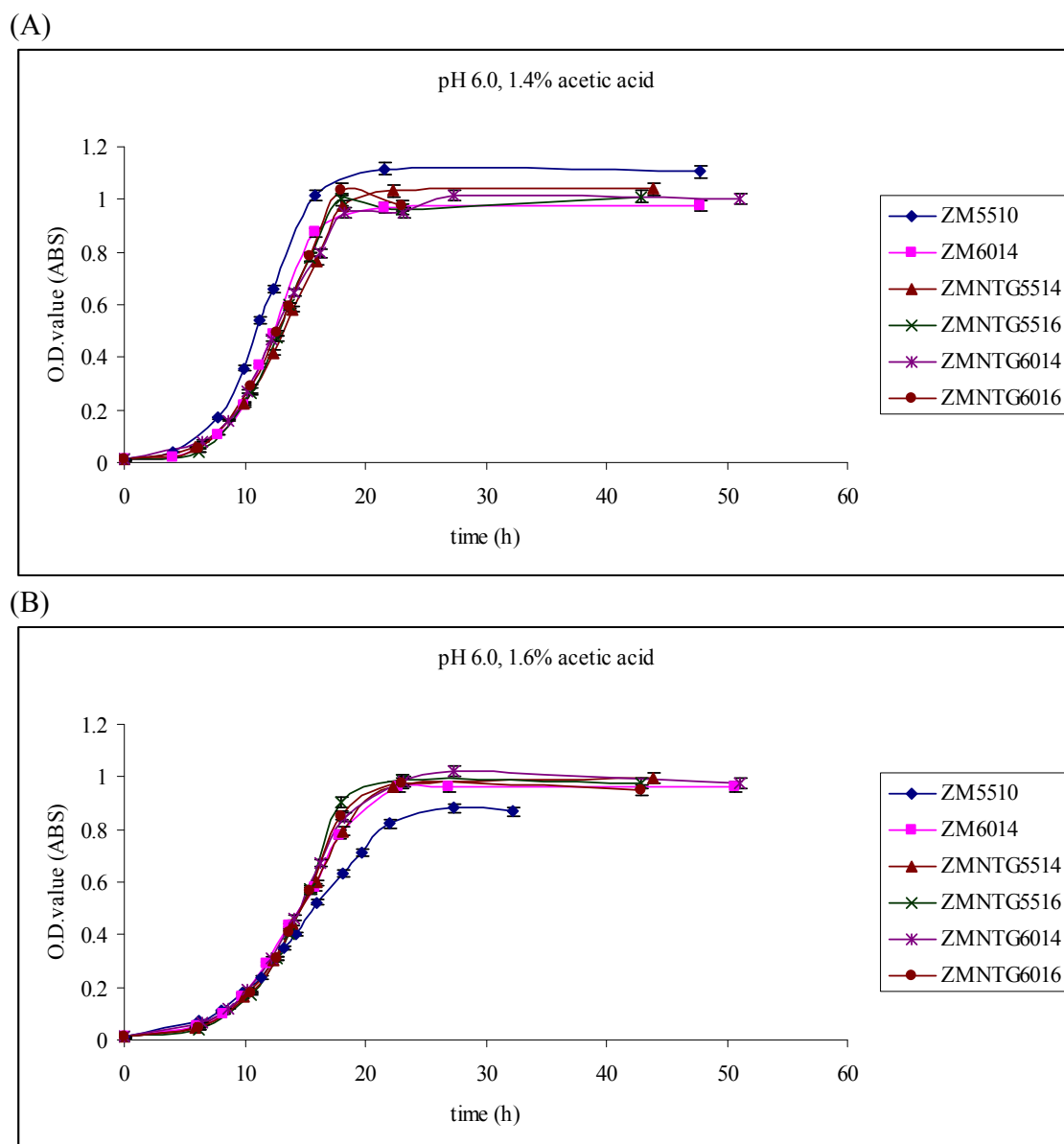


Fig. 6.13 Growth curves at pH 6.0 in the presence of (A) 1.4% and (B) 1.6% acetic acid

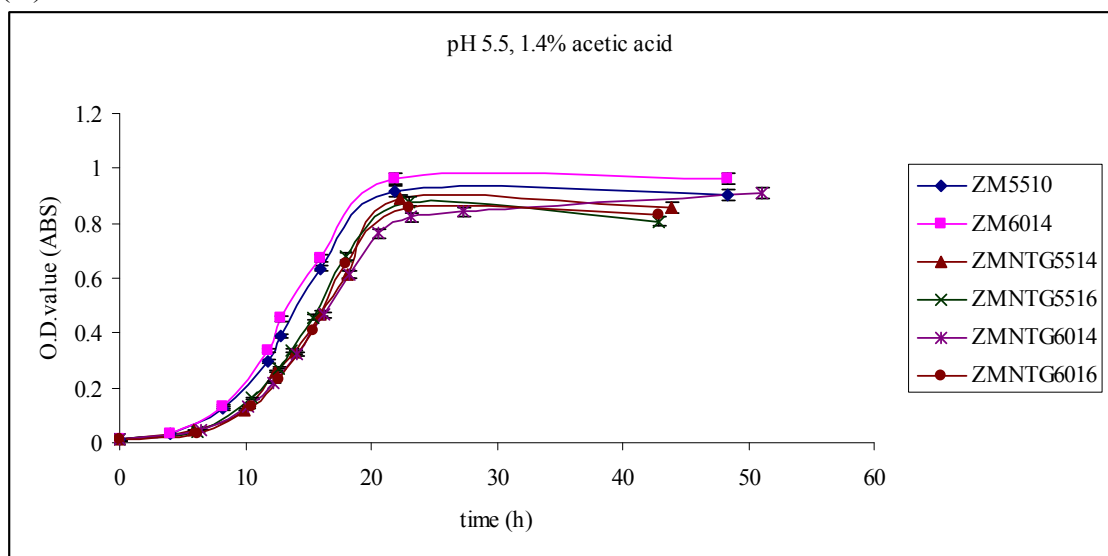
At pH 5.5 (Figure 6.14), in the presence of 1.4% acetic acid, the performances of all the mutants are similar based on the final O.D., specific growth rate (0.25 h^{-1}), and lag phase (10.2 hours). In the presence of 1.6% acetic acid, ZM5510 has the lowest specific growth rate (0.17 h^{-1}) and O.D. value. And it also has the longest lag phase (14.3 hours). Other mutants have a similar lag phase (12.5 hours), but ZMNTG5514 and ZMNTG6016

also show a slightly lower O.D. than ZM6014, ZMNTG5516 and ZMNTG6014.

ZMNTG5514, ZMNTG5516 and ZMNTG6016 have the highest specific growth rate (0.23 h^{-1}).

Compared to the experimental results obtained at pH 6.0, the lag phase becomes longer and the specific growth rate is lowered, as expected, for each strain with the same amount of acetic acid.

(A)



(B)

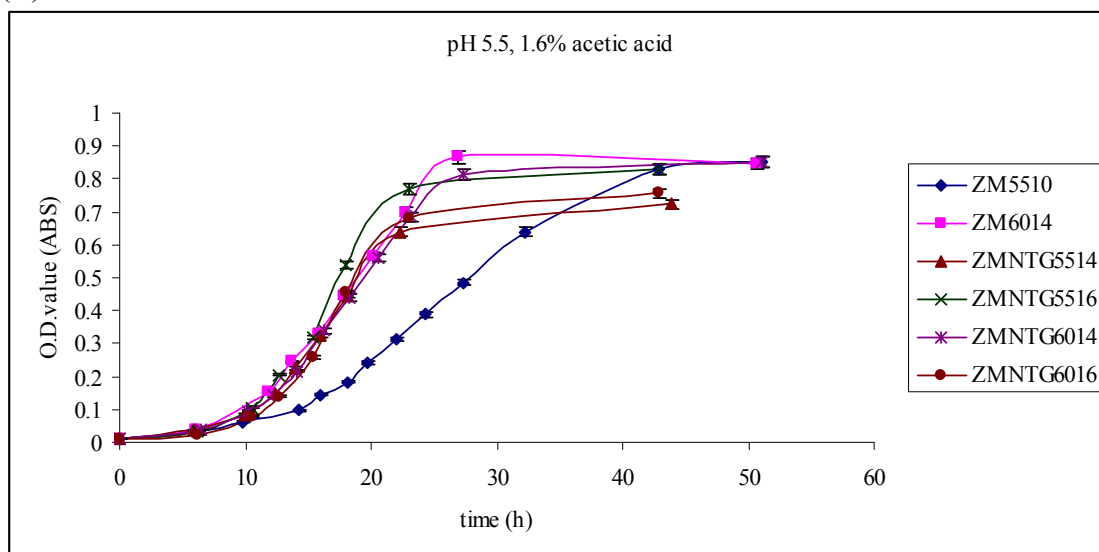
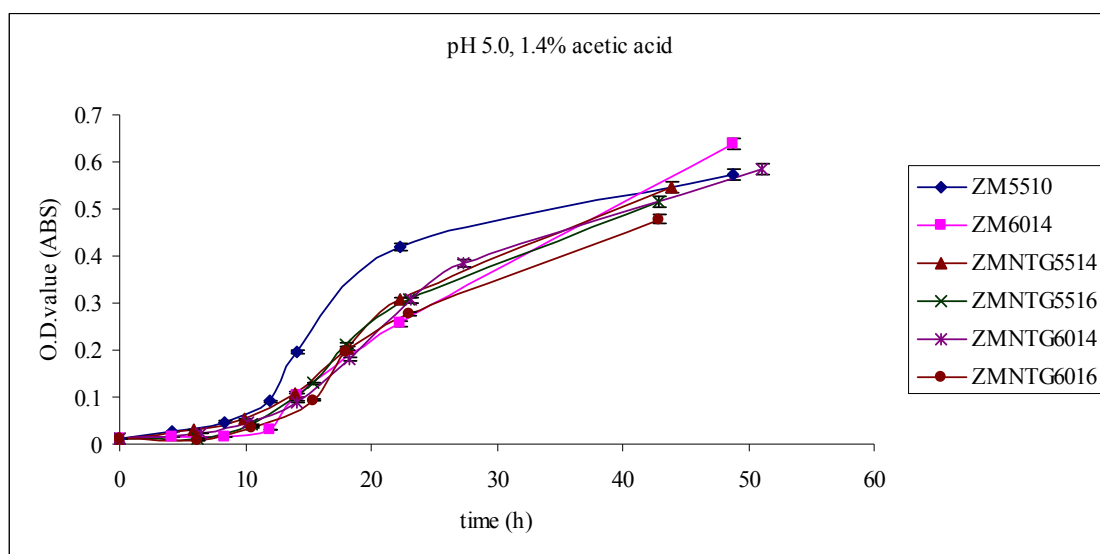


Fig. 6.14 Growth curves at pH 5.5 in the presence of (A) 1.4% and (B) 1.6% acetic acid

At pH 5.0, no strain completed the fermentation in two days (Figure 6.15). In the presence of 1.4% acetic acid, ZM5510 and ZM6014 have a slightly shorter lag phase (14.1 hours) than other mutants (14.5 hours). And ZMNTG5516 and ZMNTG6014 have a slightly higher specific growth rate (0.19 h^{-1}) than other mutants (between 0.132 and 0.164 h^{-1}). In the presence of 1.6% acetic acid, ZM5510, ZM6014 and ZMNTG6016 have longer lag phase (27.1 hours) than other mutants (22.8 hours). ZMNTG6014 has a little higher specific growth rate (0.18 h^{-1}) than other mutants. ZMNTG5514, ZMNTG5516 and ZMNTG6016, which show the highest specific growth rate at pH 5.5 in the presence of 1.6% acetic acid, have the lower specific growth rate than ZM6610. ZMNTG 5516 and ZMNTG 6016 even have the lower specific growth rate than ZM5510. This is because that the NTG mutagenesis is a random mutation, so the behaviors of the mutants obtained by NTG mutagenesis are somewhat unpredictable.

(A)



(B)

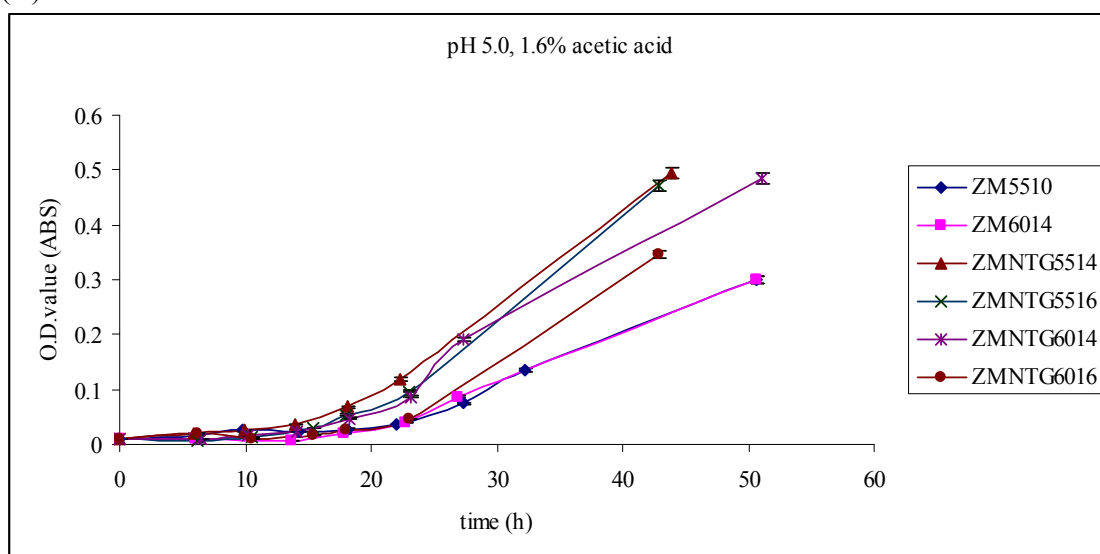


Fig. 6.15 Growth curves at pH 5.0 in the presence of (A) 1.4% and (B) 1.6% acetic acid

Table 6.3 and 6.4 summarized the lag phases and specific growth rates of all mutants at different experimental conditions.

Table 6.3 Lag phases (h) of six strains at different pHs and acetic acid concentrations

pH 5.0	Acetic acid concentration	
	1.40%	1.60%
ZM5510	14.1±0.10	27.1±0.11
ZM6014	14.1±0.10	27.1±0.11
ZMNTG5514	14.5±0.08	22.8±0.12
ZMNTG5516	14.5±0.08	22.8±0.12
ZMNTG6014	14.5±0.08	22.8±0.12
ZMNTG6016	14.5±0.08	27.1±0.11
pH 5.5	Acetic acid concentration	
	1.40%	1.60%
ZM5510	10.2±0.14	14.3±0.11
ZM6014	10.2±0.14	12.5±0.13
ZMNTG5514	10.2±0.14	12.5±0.13
ZMNTG5516	10.2±0.14	12.5±0.13
ZMNTG6014	10.2±0.14	12.5±0.13
ZMNTG6016	10.2±0.14	12.5±0.13
pH 6.0	Acetic acid concentration	
	1.40%	1.60%
ZM5510	7.8±0.15	8.3±0.14
ZM6014	7.8±0.15	8.3±0.14
ZMNTG5514	7.8±0.15	8.3±0.14
ZMNTG5516	7.8±0.15	8.3±0.14
ZMNTG6014	7.8±0.15	8.3±0.14
ZMNTG6016	7.8±0.15	8.3±0.14

Table 6.4 Specific growth rates (h^{-1}) of six strains at different pHs and acetic acid concentrations

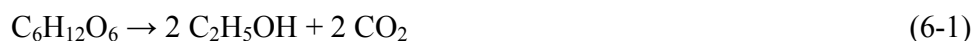
pH 5.0	Acetic acid concentration	
	1.40%	1.60%
ZM5510	0.164±0.012	0.122±0.011
ZM6014	0.149±0.004	0.135±0.017
ZMNTG5514	0.146±0.002	0.135±0.007
ZMNTG5516	0.193±0.008	0.115±0.012
ZMNTG6014	0.186±0.008	0.184±0.003
ZMNTG6016	0.132±0.011	0.112±0.010
pH 5.5	Acetic acid concentration	
	1.40%	1.60%
ZM5510	0.247±0.012	0.170±0.007
ZM6014	0.252±0.014	0.211±0.004
ZMNTG5514	0.249±0.005	0.231±0.013
ZMNTG5516	0.243±0.008	0.235±0.012
ZMNTG6014	0.239±0.005	0.208±0.020
ZMNTG6016	0.264±0.003	0.235±0.003
pH 6.0	Acetic acid concentration	
	1.40%	1.60%
ZM5510	0.331±0.018	0.249±0.024
ZM6014	0.351±0.007	0.312±0.006
ZMNTG5514	0.329±0.010	0.298±0.010
ZMNTG5516	0.330±0.007	0.322±0.012
ZMNTG6014	0.317±0.017	0.298±0.008
ZMNTG6016	0.328±0.006	0.301±0.003

In summary, it is difficult to compare the acetic acid tolerance between these mutants. Although at some experimental conditions, some ZMNTG mutants show better performance than ZM5510 or ZM6014 based on one of those three parameters, these mutants could also show worse performances under other experimental conditions. No ZMNTG mutant shows the better performance than ZM6014 in all experimental conditions. This means adaptive mutation alone is sufficient to develop the acetic acid tolerant strain and further treatment with NTG does not lead to better mutants.

6.3 Ethanol production characteristics of mutants

The growth of the mutants is important. More important is the ethanol production characteristics. If the mutants could grow in the high acetic acid environment but has unfavorable ethanol production characteristics, such as low ethanol yield, these mutants would be useless.

Based on the stoichiometry, the theoretical ethanol yield should be 50%. That means the ethanol concentration should be around 2.5% if the initial glucose concentration is 5%.



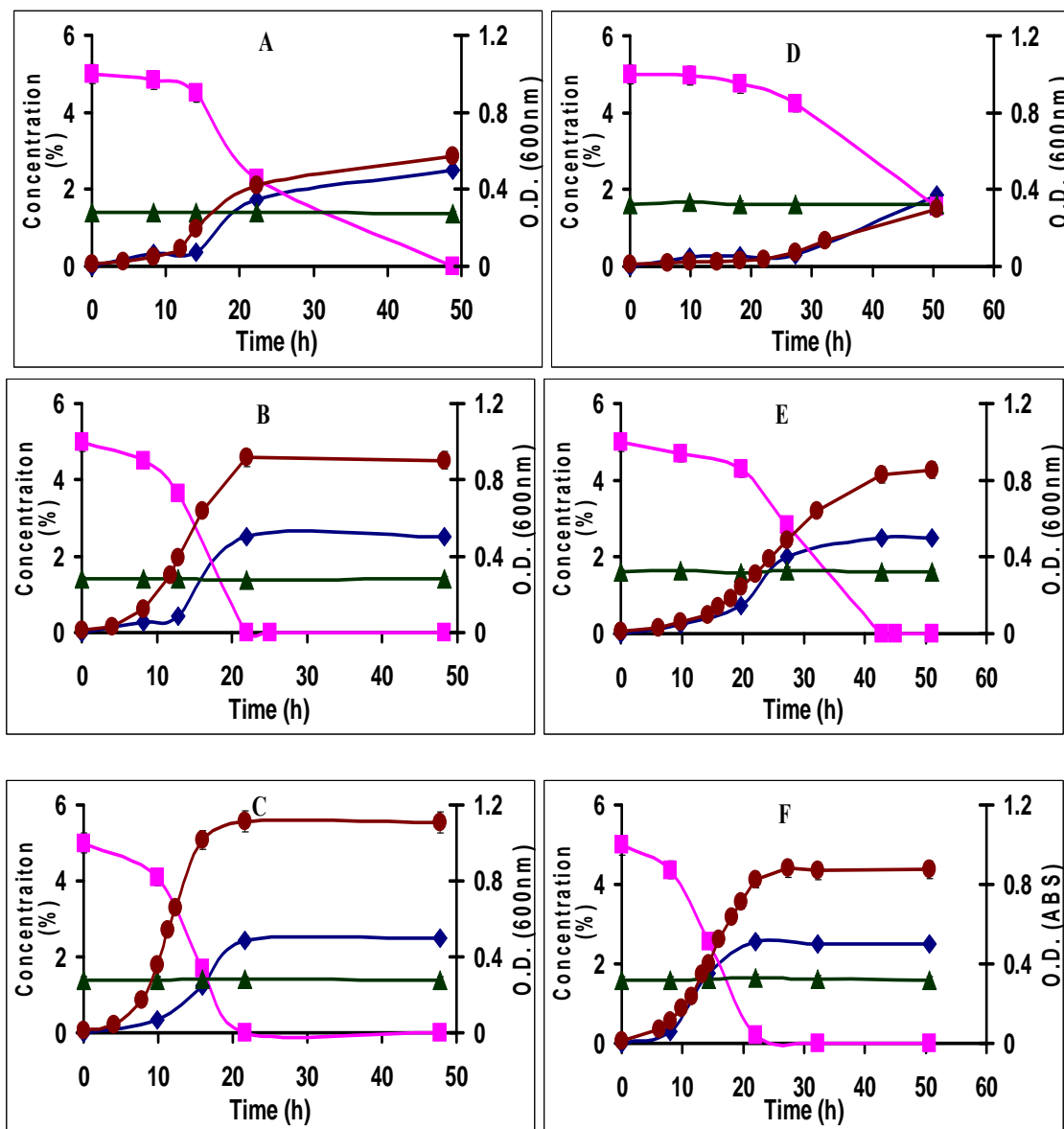


Fig. 6.16 Ethanol production of ZM5510 at different pHs and concentrations. (A) pH 5.0, 1.4% acetic acid, (B) pH 5.5, 1.4% acetic acid, (C) pH 6.0, 1.4% acetic acid, (D) pH 5.0, 1.6% acetic acid, (E) pH 5.5, 1.6% acetic acid, (F) pH 6.0, 1.6% acetic acid. —◆— Ethanol, —■— Glucose, —▲— Acetic acid, —●— O.D.

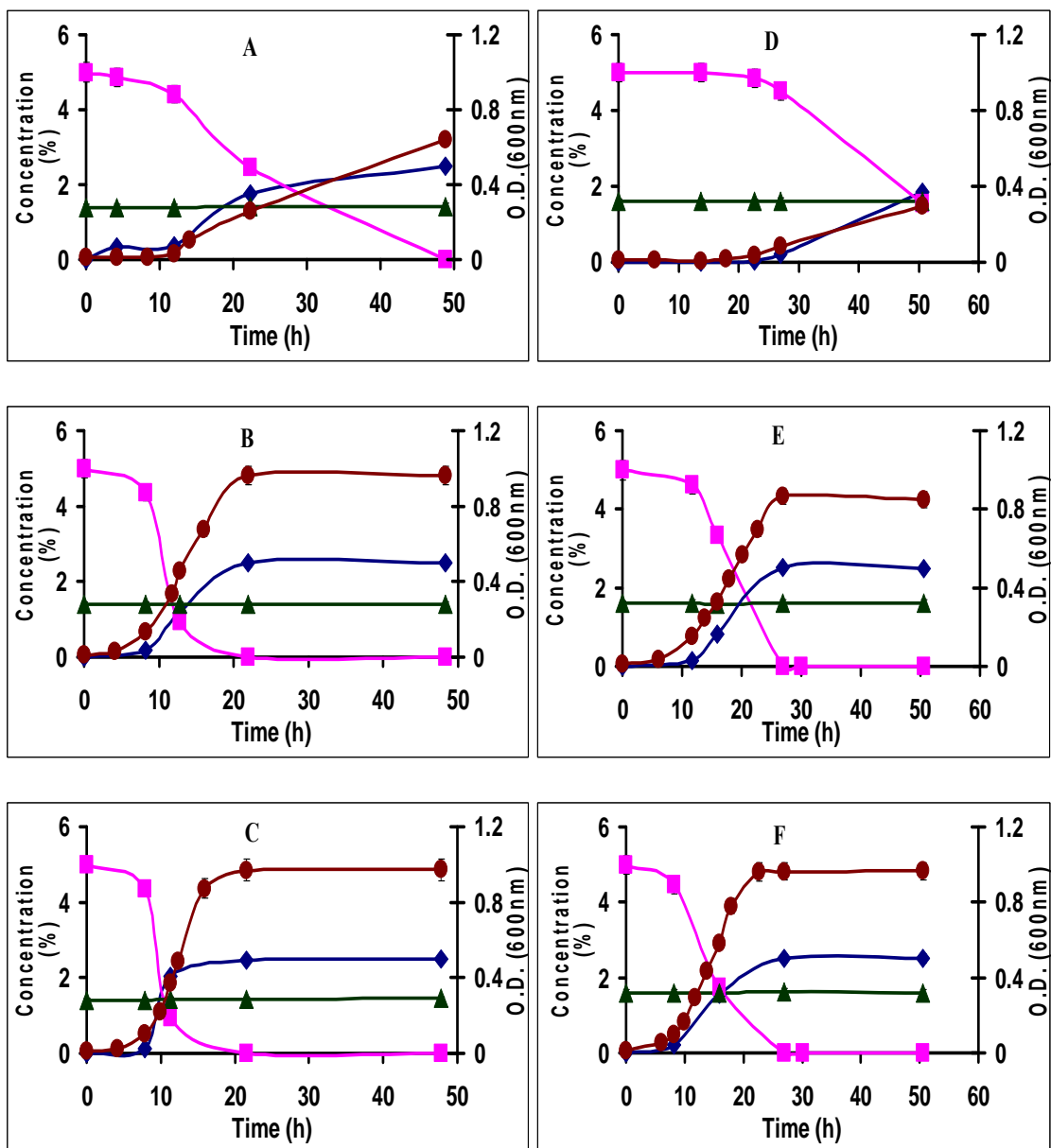


Fig. 6.17 Ethanol production of ZM6014 at different pHs and concentrations. (A) pH 5.0, 1.4% acetic acid, (B) pH 5.5, 1.4% acetic acid, (C) pH 6.0, 1.4% acetic acid, (D) pH 5.0, 1.6% acetic acid, (E) pH 5.5, 1.6% acetic acid, (F) pH 6.0, 1.6% acetic acid. —◆— Ethanol, —■— Glucose, —▲— Acetic acid, —●— O.D.

Figure 6.16 and 6.17 show the ethanol production, glucose consumption and the acetate concentration, as well as the growth curve as a function of time. The strain was ZM5510 and ZM6014, which were stated as the best mutants obtained from adaptive mutation. In all experimental conditions, the initial glucose is about 5%. The strain does

not start to ferment the glucose immediately, which is corresponding to the lag phase in the growth. Once the strain reaches the maximum O.D., the glucose concentration is reduced to zero. The ethanol concentration curve shows that the strain started to produce ethanol as soon as this strain started to consume the glucose. The final ethanol concentration is around 2.5%, if the glucose concentration is zero. In conclusion, the ethanol production is related to the cell growth. There is a similar lag phase between ethanol production (glucose consumption) and cell growth. The time needed for ethanol production is equal to the time needed for cell to reach the maximum biomass.

The acetic acid concentrations were also measured throughout the fermentation. The acetic acid curves show that it is constant with time. This shows that consumption of acetic acid is not the tolerant mechanism. The pH measured at the end of fermentation was the same as the initial value, indicating constant pH during the fermentation. The constant pH and acetic acid suggest that the tolerance is not due to the change of environmental conditions during fermentation..

The ethanol yields of these two mutants were also evaluated under other conditions. Results are summarized in Table 6.5. The experimental results showed that once the fermentation was completed, the ethanol yield was always around the theoretical value, which was 50%. Ethanol yield of fermentation obtained at pH 5.0 in the presence of 1.6% acetic acid was only about 66% of the theoretical value. This is due to the incomplete fermentation within two days. However the experiments carried out under other conditions were all completed in two days, the final yields were all about 100% of the theoretical value. This means mutant ZM5510 and ZM6014, retain the advantage of high ethanol yield.

Table 6.5 Ethanol production characteristics of two mutants in different fermentation conditions (% of theoretical number)

	ZM5510	ZM6014
5014	102.0±1.99	100.1±0.73
5514	99.6±1.37	99.5±1.04
6014	97.9±1.48	100.7±0.72
5016	65.8±1.74	67.2±0.97
5516	99.3±0.84	98.6±1.29
6016	99.6±1.16	100.1±0.60

6.4 Other inhibitors

To test whether mutants developed for acetic acid tolerance could cross-protect the cells from other inhibitors, additional experiments were carried out with four common inhibitors (vanillin, formic acid, hydroxybenzoic acid, and furfuryl alcohol) derived from biomass. Each inhibitor was evaluation at two concentrations.

The mutant ZM6014 was chosen for the study. The O.D. values after two day fermentation and the specific growth rates are summarized in Table 6.6. The ethanol productions after two day fermentation are summarized in Table 6.7.

Mutant ZM6014 shows the higher tolerance to formic acid than wild type strain. In the presence of 2.68 g/l formic acid, ZM6014 grows to a much higher O.D. and specific growth rate than wild type strain. It is reasonable, because the formic acid is a weak acid. The mechanism of tolerance of formic acid should be similar to acetic acid, which is also a weak acid.

The mutant ZM6014 also shows the higher tolerance to vanillin and hydroxybenzoic acid based on specific growth rate, especially in the presence of high concentration of

vanillin (1 g/L) and hydroxybenzoic acid (6.8 g/L). Vanillin and hydroxybenzoic acid are the inhibitors belonging to the phenolic compounds generated due to lignin breakdown and also carbohydrate degradation during acid hydrolysis. Inhibition mechanisms of phenolic compounds have not yet been completely elucidated, but was believed to act on biological membranes, causing loss of integrity, thereby affecting their ability to serve as selective barriers and enzyme matrices [80]. One possible mechanism of acetic acid tolerance is the alterations of cell membrane as stated in literature review, so this maybe the reason that increases the tolerance to phenolic compounds.

Furfuryl alcohol is a furan derivative formed by dehydration of hexoses. There is no inhibition observed in concentration range in our experiments.

Table 6.6 O.D. value and specific growth rate of fermentation in the presence of different inhibitors

Inhibitors	Final O. D.		Specific growth rate (h⁻¹)	
	Wild type ZM4	ZM6014	Wild type ZM4	ZM6014
Control (no inhibitor)	1.5365±0.011	1.4905±0.019	0.507±0.012	0.534±0.019
Vanillin (0.5g/L)	1.310±0.064	1.364±0.071	0.231±0.001	0.290±0.002
Vanillin (1g/L)	1.013±0.037	1.111±0.082	0.168±0.027	0.242±0.001
Formic acid (2.68g/L)	0.546±0.036	0.927±0.024	0.208±0.003	0.2973±0.004
Formic acid (5.37g/L)	0.021±0.000	0.027±0.000	N/A	N/A
Hydroxybenzoic acid (3.4g/L)	1.467±0.045	1.421±0.014	0.405±0.019	0.428±0.030
Hydroxybenzoic acid (6.8g/L)	1.446±0.028	1.476±0.029	0.368±0.010	0.388±0.008
Furfuryl alcohol (3.89g/L)	1.469±0.027	1.444±0.027	0.507±0.019	0.510±0.004
Furfuryl alcohol (7.79g/L)	1.468±0.005	1.454±0.024	0.510±0.008	0.536±0.015

The ethanol yields are not affected by the inhibitors. Once the fermentation finished, and the strain reached the maximum O.D., the ethanol yield is always similar to the theoretical number. The ethanol production of strain is only 5% of theoretical value in the presence of 5.37 g/L formic acid. This is because the strain only grows a little during experimental time.

**Table 6.7 Ethanol production of two strains in the presence of different inhibitions
(% of theoretical number)**

Inhibitors	Wild type ZM4	ZM6014
Control (no inhibitor)	98.6±2.55	99.7±1.34
Vanillin (0.5g/L)	101.0±1.20	99.2±1.65
Vanillin (1g/L)	99.5±2.25	102±1.44
Formic acid (2.68g/L)	98.9±2.30	97.0±1.79
Formic acid (5.37g/L)	5.1±0.000	5.2±0.000
Hydroxybenzonic acid (3.4g/L)	97.7±1.03	98.5±1.65
Hydroxybenzonic acid (6.8g/L)	98.0±2.34	99.4±1.89
Furfuryl alcohol (3.89g/L)	98.7±2.46	99.6±2.63
Furfuryl alcohol (7.79g/L)	98.3±1.70	99.9±2.39

CHAPTER 7 CONCLUSIONS AND RECOMMENDATIONS

In this chapter, important results will be summarized. Recommendations on how to improve the experiments will be presented. Finally, future research in using this method will be proposed.

7.1 Conclusions

Although *Zymomonas mobilis* was proved to have high ethanol yield, high ethanol tolerance, high glucose tolerance and high glucose uptake rate, its application in industry was hampered by its low acetic acid tolerance. Acetic acid is an inhibitor that affects cell growth. Our experimental results showed that, regardless pH, higher acetic acid concentration resulted in lower specific growth rate and lower produced biomass and longer lag phase. The effect of acetic acid became much more severe at low pH.

Previous strategies used to reduce the inhibition of acetic acid include recombinant, DNA technology, random mutation and process optimization. Random mutagenesis is the only method, which has led the strain with significant acetic acid tolerance. In this study, through adaptive mutation, several useful acetic acid tolerant strains were developed. The characteristics of these mutants were carefully studied. Compared to the wild type strain, these mutants exhibited higher specific growth rate, higher final O.D. and had significantly shorter lag phase in the presence of acetic acid, indicating superior tolerance for the mutants. For example, the best mutant could grow at the most inhibitive condition tested (pH 5.0 and 1.4% acetic acid concentration) with specific growth rate 0.16 h^{-1} , whereas the wild type strain could not grow at all under the same condition.

ZM5510, one of the mutants, was further treated by NTG, resulting four new mutants. Subsequent studies, however, did not find convenient evidence for superior tolerance for these mutants. It appears that the adaptive mutation alone is enough to develop the acetic acid tolerant strains.

Importantly, mutants retained high ethanol fermentation capability, with ethanol yield approaching the theoretical yield. Ethanol fermentation time profile and the lag phase correspond to the cell growth, which indicates tight coupling of the ethanol production with cell growth.

This study also reveals that acetic acid tolerance lead to tolerance to other biomass derived inhibitors including formic acid, hydroxybenzoic acid, and vanillin. This cross-protection makes acetic acid tolerant strains more attractive for use in bioethanol production from renewable sources.

In summary, by adaptive mutation, the acetic acid tolerant *Zymomonas mobilis* strains were successfully developed. These strains have been proved to have higher acetic acid tolerance than wild type strain and still have the high ethanol yield. These mutants have also been proved to have higher tolerance to other inhibitors, such as formic acid, vanillin and hydroxybenzoic acid.

7.2 Recommendation

Although the acetic acid tolerant mutants were successfully developed by adaptive mutation, there are opportunities to fine-tune the process.

First, the number of the agar plates could be increased. Obviously, the more single colonies grow on the agar plates, the more choices we have when picking single colonies.

Second, the increment of acetic acid concentration could be reduced. This will allow the strain to better adapt the selective pressure, acetic acid, more gradually. Hopefully, the better mutants could be obtained by doing this. The improvement of the procedure could lead to the better mutants.

7.3 Future research

The availability of acetic acid tolerant *Z. mobilis* strains offers opportunity to probe the mechanism of tolerance in this microorganism.

Previous research has proposed several possibilities for an microorganism to tolerate acetic acid. These include the increased expression of certain gene products related to the maintenance of intracellular pH, biosynthesis of fatty acids of cell membrane, protection of proteins from denaturizing and consumption of acetic acid by TCA cycle. These mechanisms were known to exist in the naturally acetic acid tolerant microorganisms, but no research has been done on the mechanism of *Z. mobilis*. Although NTG mutagenesis has been proved as a useful method to increase the acetic acid tolerance of *Z. mobilis*, these mutants were not ideal for mechanism research, because of its random nature. The mutants developed by random mutagenesis could contain multiple changes in the genome, some of which may be not related to acetic acid tolerance.

The mutants developed by adaptive mutation are better suited for this type of research. During adaptive mutation process, only mutations beneficial to tolerance occur. A variety of techniques could be used to identify the genetic alterations responsible for the tolerance. These include gene expression profiling to identify expression genes

associated with tolerance and membrane analysis to investigate changes at the fatty acid level.

Another research direction is to use adaptive mutation for other inhibitors. Several inhibitors exist in the pretreated biomass and they belong to different categories. Our research showed that adapting a strain to acetic acid could increase the tolerance of this strain to other weak acid (formic acid). Similar adaptive mutation could be used to develop tolerance to vanillin or other inhibitors directly, in sequential manner or in combination. The tolerance to common biomass-derived inhibitors will enhance the benefit of this strain for ethanol production.

REFERENCES

1. *Fossil fuel*. http://en.wikipedia.org/wiki/Fossil_fuel, March 2008.
2. Ngo, A.-T. and G. West, *Advantages and disadvantages of using ethanol: the consumer viewpoint*. AIE A2 International conference and workshop of the CAES, 2004.
3. *Ethanol*. <http://www.ethanol-gec.org/clean/cf03.htm>, March 2008.
4. *Bioethanol*. <http://www.vermontbiofuels.org/biofuels/ethanol.shtml>, March 2008.
5. Almeida, J.R.M., et al., *Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by Saccharomyces cerevisiae*. Journal of chemical technology and biotechnology 2007. **82**(4): p. 340-349.
6. Desimone, M.F., et al., *Ethanol tolerance in free and sol-gel immobilised Saccharomyces cerevisiae*. Biotechnology letters, 2002. **24**: p. 1557-1559.
7. You, K.M., C.-L. Rosenfield, and D.C. Knipple, *Ethanol tolerance in the Yeast Saccharomyces cerevisiae is dependent on cellular oleic acid content*. Applied and environmental microbiology, 2003. **69**(3): p. 1499-1503.
8. Mobini-Dehkordi, M., et al., *Isolation of high ethanol resistant strains of Saccharomyces cerevisiae*. Research in pharmaceutical sciences, 2007. **2**: p. 85-91.
9. Bazua, C.D. and C.R. Wilke, *Ethanol effects on the kinetics of a continuous fermentation with Saccharomyces cerevisiae*. ERDA energy resource, 1976.
10. Nissen, T., et al., *Anaerobic and aerobic batch cultivations of Saccharomyces cerevisiae mutants impaired in glycerol synthesis*. Yeast, 2000. **16**(5): p. 463-474.
11. Visser, W., et al., *Oxygen requirement of yeasts*. Applied and environmental microbiology, 1990. **56**(12): p. 3785-3792.
12. Bideaux, C., et al., *Minimization of glycerol production during the high-performance fed-batch ethanolic fermentation process in Saccharomyces cerevisiae, using a metabolic model as a prediction tool*. Applied and environmental microbiology, 2006. **72**(3): p. 2134-2140.
13. Nagodawithana, T.W., C. Castellano, and K.H. Steinkraus, *Effect of dissolved oxygen, temperature, initial cell count, and sugar concentration on the viability of Saccharomyces cerevisiae*. Applied microbiology, 1974. **28**(3): p. 383-391.
14. Wyman, C., *Handbook on bioethanol: production and utilization*. 1996.

15. Jeffries, et al., *Xylose-fermenting recombinant yeast strains*, U.p.a.t. office, Editor. 2006.
16. Maris, A.J.V., et al., *Development of efficient xylose fermentation in Saccharomyces cerevisiae: xylose isomerase as a key component*. Advanced biochemical engineering and biotechnology, 2007. **108**: p. 179-204.
17. Eckhard, B., et al., *Arabinose- and xylose-fermenting Saccharomyces cerevisiae strains*, E.p. office, Editor. 2006.
18. Dien, B.S., M.A. Cotta, and T.W. Jeffries, *Bacteria engineered for fuel ethanol production: current status*. Applied microbiol biotechnology, 2003. **63**: p. 258-266.
19. Lee, K.J., et al., *High productivity ethanol fermentations with Zymomonas mobilis using continuous cell recycle*. Biotechnology letters, 1980. **2**(11): p. 487-492.
20. Rogers, P.L., K.J. Lee, and D.E. Tribe, *High productivity ethanol fermentations with Zymomonas mobilis*. Process Biochemistry (Rickmansworth, United Kingdom), 1980. **15**(6): p. 7-11.
21. Rogers, P.L., et al., *Ethanol fermentation by highly productive strains of Zymomonas mobilis*. Adv. Biotechnol., [Proc. Int. Ferment. Symp.] 6th, 1980.
22. El-Mansi, M., *Fermentation microbiology and biotechnology*. 2007: CRC Press.
23. fraser-reid, B., K. tatsuta, and J. thiem, *Glycoscience: Chemistry and Chemical Biology*. 2001: Springer.
24. Panesar, P.S., S.S. Marwaha, and J.F. Kennedy, *Zymomonas mobilis: an alternative ethanol producer*. Journal of chemical technology and biotechnology, 2006. **81**: p. 623-635.
25. Rogers, P.L., K.L. Lee, and D.E. Tribe, *Kinetics of alcohoh production by Zymomonas mobilis at high sugar concentrations*. Biotechnology letter, 1979. **1**: p. 165-170.
26. Pronk, J.T. and W.A. Scheffers, *Alcoholic fermentation of carbon sources in biomass hydrolysates by Saccharomyces cerevisiae: current status*. Atonic van leeuwenhoek, 2006. **90**: p. 391-418.
27. Zhang, M., *Engineering Zymomonas mobilis for efficient ethanol production from lignocellulosic feedstocks*. ACS national meeting, 2003.
28. Zhang, M., et al., *Zymomonas pentose-sugar fermenting strains and uses thereof*, U.S. patant, Editor. 2003.

29. Mohagheghi, A., et al., *Cofermentation of glucose, xylose, and arabinose by genomic DNA-integrated xylose/arabinose fermenting strain of Zymomonas mobilis AX101*. Applied biochemistry and biotechnology, 2002. **98-100**: p. 885-898.
30. Lawford, H.G. and J.D. Rousseau, *The effect of acetic acid on fuel ethanol production by Zymomonas*. Applied biochemistry and biotechnology, 1993. **39/40**: p. 687-699.
31. Jeon, Y.J., et al., *Kinetic analysis of ethanol production by an acetate-resistant strain of recombinant Zymomonas mobilis*. Biotechnology letters, 2002. **25**: p. 819-824.
32. Joachimasthal, E., et al., *A mutant of Zymomonas mobilis ZM4 capable of ethanol production from glucose in the presence of high acetate concentrations*. Biotechnology letter, 1998. **20(2)**: p. 137-142.
33. Kim, I.S., K.D. Barrow, and P.L. Rogers, *Nuclear magnetic resonance studies of acetic acid inhibition of Rec Zymomonas mobilis ZM4 (pZB5)*. Applied biochemistry and biotechnology, 2000. **84-86**.
34. Baumler, D.J., et al., *Enhancement of acid tolerance in Zymomonas mobilis by a proton-buffering peptide*. Applied biochemistry and biotechnology, 2006. **134**: p. 15-26.
35. Han, B., et al., *Adsorptive membranes vs. resins for acetic acid removal from biomass hydrolysates*. Desalination, 2006. **193**: p. 361-366.
36. McMillan, J.D., *Conversion of hemicellulose hydrolysates to ethanol*. . Enzymatic conversion of biomass for fuels production, 1994. **566**: p. 411-437.
37. Lawford, H.G., J.D. Rousseau, and J.D. McMillan, *Optimization of Seed Production for a Simultaneous Saccharification Cofermentation Biomass-to-Ethanol Process Using Recombinant Zymomonas*. Applied Biochemistry and Biotechnology, 1997. **63-65**: p. 269-286.
38. http://en.wikipedia.org/wiki/Adaptive_mutation, March 2008.
39. Rosenberg, S.M., R.S. Harris, and J. Torkelson, *Molecular handles on adaptive mutation*. Molecular microbiology, 1995. **18(2)**: p. 185-189.
40. Williams, A.B. and P.L. Foster, *The Escherichia coli histone-like protein HU has a role in stationary phase adaptive mutation*. Genetics, 2007. **177**: p. 723-735.
41. Foster, P.L., *Mechanisms of stationary phase mutation: a decade of adaptive mutation*. Annual review of genetics, 1999. **33**: p. 57-88.

42. Foster, P.L., *Adaptive mutation: the uses of adversity*. Annual reviews of microbiology, 1993. **47**: p. 467-504.
43. Osman, Y.A. and L.O. Ingram, *Zymomonas mobilis mutants with an increased rate of alcohol production*. Applied and environmental microbiology, 1987. **53**(7): p. 1425-1432.
44. Delgenes, J.P., R. Moletta, and J.M. Navarro, *Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stipitis, and Candida shehatae*. Enzyme and microbial technology, 1996. **19**: p. 220-225.
45. Murooka, Y. and T. Imanaka, *Recombinant microbes for industrial and agricultural applications*. 1994: CRC press.
46. Gunasekaran, P., T. Karunakaran, and M. Kasthuribai, *Fermentation pattern of Zymomonas mobilis strains on different substrates-a comparative study*. Journal of bioscience, 1986. **10**(2): p. 181-186.
47. *Zymomonas mobilis*. http://en.wikipedia.org/wiki/Zymomonas_mobilis, March 2008.
48. Rogers, P.L., et al., *Ethanol production by Zymomonas mobilis*. Advances in biochemical engineering, 1982. **23**: p. 37-84.
49. Doelle, H.W., *Microbial process Development*. 1994: World scientific.
50. Robert, K.P., *Advances in microbial physiology*. 2006: Academic press.
51. Swings, J. and J.D. Ley, *The biology of Zymomonas*. Bacteriological reviews, 1977. **41**(1): p. 1-46.
52. Robert, M.P., *The evolution of metabolic function*. 1992: CRC press.
53. Gunasekaran, P. and K.C. Raj, *Ethanol fermentation technology - Zymomonas mobilis*. <http://www.ias.ac.in/currsci/jul10/articles14.htm>, March 2008.
54. Carry, V.C. and L.O. Ingram, *Lipid composition of Zymomonas mobilis: effects of ethanol*. Journal of bacteriology, 1983. **154**(3): p. 1291-1300.
55. Toran-Diaz, I., et al., *Effect of acid or enzymatic hydrolysis on ethanol production by zymomonas mobilis growing on jerusalem artichoke juice*. Biotechnology letter, 1985. **7**(7): p. 527-530.
56. Duvnjak, Z., et al., *Production of alcohol from Jerusalem artichokes by yeasts*. Biotechnology and bioengineering, 1982. **24**(11): p. 2297-2308.

57. Lawford, H.G. and L.S. Lacis, *Thermoanaerobacter ethanolicus* growth and product yield from elevated levels of xylose or glucose in continuous cultures. Applied and environmental microbiology, 1991. **57**(2): p. 579-585.
58. Torres, E. and J. Baratti, *Ethanol production from wheat flour by Zymomonas mobilis*. Journal of fermentation technology, 1988. **66**: p. 167-172.
59. Nellaiah, H., T. Karunakaran, and P. Gunasekaran, *Ethanol fermentation of cassava starch by Zymomonas mobilis NRRL B-4286*. Biomass, 1988. **15**(3): p. 201-207.
60. Allias, J.-J., E.F. Torres, and J. Baratti, *Continuous production of ethanol with Zymomonas mobilis growing on Jerusalem artichoke juice*. Biotechnology and bioengineering, 1987. **29**(6): p. 778-782.
61. Richards, L. and H.W. Doelle, *Fermentation of potato mash, potato mash/maltin mixtures, maltin and wheat starch*. World journal of microbiology & biotechnology, 1989. **5**(3): p. 307-312.
62. Sahm, H. and S. Bringer-Meyer, *Continuous ethanol production by Zymomonas mobilis on an industrial scale*. Acta biotechnology, 1987. **7**(4): p. 307-313.
63. Amin, G., *Conversion of sugar-beet particles to ethanol by the bacterium Zymomonas mobilis in solid-state fermentation*. Biotechnology letter, 1992. **14**(6): p. 499-504.
64. Dale, M.C. and M. Moelhman, *Enzymatic simultaneous saccharification and fermentation (SSF) of biomass to ethanol in a pilot 130 liter multistage continuous reactor separator*.
65. Rhee, H.-K., I.-S. Nam, and J.M. Park, *New developments and application in chemical reaction engineering*. 2006: Elsevier.
66. *Acetic acid*. http://en.wikipedia.org/wiki/Acetic_acid, March 2008.
67. Lawford, H.G., *Effects of pH and acetic acid on glucose and Xylose metabolism by a genetically engineered ethanologenic Escherichia coli*. Applied biochemistry and biotechnology, 1993. **39/40**: p. 301-322.
68. Takahashi, C.M., *Effects of acetate on the growth and fermentation performance of Escherichia coli KO11*. Applied biochemistry and biotechnology, 1999. **81**: p. 193-203.
69. Note, S., *The pH-dependent energetic uncoupling of Zymomonas by acetic acid*. Applied biochemistry and biotechnology, 1994. **45/46**: p. 437-447.
70. *Denaturation* [http://en.wikipedia.org/wiki/Denaturation_\(biochemistry\)](http://en.wikipedia.org/wiki/Denaturation_(biochemistry)), March 2008.

71. Ferguson, R.A. and R.G. Boutilier, *Metabolic-membrane coupling in red blood cells of trout: the effect of anoxia and adrenergic stimulation*. Journal of external biology, 1989. **143**: p. 149-164.
72. Saez-Miranda, J.C., L. Saliceti-Piazza, and J.D. McMillan, *Measurement and Analysis of Intracellular ATP Levels in Metabolically Engineered Zymomonas mobilis Fermenting Glucose and Xylose Mixtures*. Biotechnology Progress, 2006. **22**: p. 359-368.
73. Fukaya, M., et al., *Cloing of genes responsible acetic acid resistance in Acetobacter aceti*. Journal of bacteriology, 1990. **172**: p. 2096-2104.
74. Fukaya, M., et al., *The aarC gene responsible for acetic-acid assimilation confers actic-acid resistance on Acetobacter Aceti*. . Journal of fermentation technology, 1993. **76**: p. 270-275.
75. Lasko, D.R., et al., *Acetate-specific stress response in acetate-resistant bacteria: an analysis of protein patterns*. Biotechnology Progress, 1997. **13**: p. 519-523.
76. Arnold, C.N., et al., *Global analysis of Escherichia coli gene expression during the acetate-induced acid tolerance response*. Journal of bacteriology, 2001. **183**(7).
77. Paula, S., A.G. Volkov, and D.W. Deamer, *Permeation of halide anions through phospholipid bilayers occurs by the solubility-diffusion mechanism*. Biophysical journal, 1998. **74**: p. 319-327.
78. Yu, J. and J. Wang, *Metabolic flux modeling of detoxification of acetic acid by Ralstonia eutropha at slightly alkaline pH levels*. 2000.
79. *Exponential growth*. http://en.wikipedia.org/wiki/Exponential_growth, March 2008.
80. Almeida, J.R., et al., *Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by Saccharomyces cerevisiae*. Journal of chemical technology and biotechnology, 2007. **82**: p. 340-349.